Endogenous mono-ADP-ribosylation of the free G$\beta\gamma$ prevents stimulation of phosphoinositide 3-kinase-γ and phospholipase C-β2 and is activated by G-protein-coupled receptors

Rosita LUPI*, Nadia DANI*, Alexander DIETRICH†[1], Adriano MARCHEGIANI*, Sabrina TURACCHIO*, Christopher P. BERRIE*, Joel MOSS‡, Peter GIERSCHIK†, Daniela CORDA*[2] and Maria DI GIROLAMO*[2]

*Department of Cell Biology and Oncology, Istituto di Ricerche Farmacologiche 'Mario Negri', Consorzio Mario Negri Sud, Via Nazionale, 66030 Santa Maria Imbaro (Chieti), Italy, †Department of Pharmacology and Toxicology, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany, and ‡Pulmonary-Critical Care Medicine Branch, National Institutes of Health, Bethesda, MD, U.S.A.

We have recently demonstrated that the β subunit of the heterotrimeric G-proteins is endogenously mono-ADP-ribosylated in intact cells. The modified $\beta\gamma$ heterodimer loses its ability to inhibit calmodulin-stimulated type 1 adenylate cyclase and, remarkably, is de-ADP-ribosylated by a cytosolic hydrolase that completes an ADP-/de-ADP-ribosylation cycle of potential physiological relevance. In the present study, we show that this ADP-ribosylation might indeed be a general mechanism for termination of $\beta\gamma$ signalling, since the ADP-ribosylated $\beta\gamma$ subunit is also unable to activate both phosphoinositide 3-kinase-γ and phospholipase C-β2. Moreover, we show that β subunit ADP-ribosylation is induced by G-protein-coupled receptor activation, since hormone stimulation of Chinese-hamster ovary plasma membranes leads to increases in β subunit labelling. This occurs when $\beta\gamma$ is in its active heterodimeric conformation, since full inhibition of this modification can be achieved by binding of GDP-αi3 to the $\beta\gamma$ heterodimer. Taken together, these findings delineate a pathway that arises from the activation of a G-protein-coupled receptor and leads to the inhibition of $\beta\gamma$ activity through its reversible mono-ADP-ribosylation.

Key words: CHO cell, mono-ADP-ribosyltransferase.

INTRODUCTION

G-protein-coupled receptors translate a variety of external physical and chemical stimuli that provoke cellular responses, thereby regulating numerous cellular functions, including apoptosis, chemotaxis, secretion, cell proliferation and differentiation [1–4]. When activated by extracellular signals, G-protein-coupled receptors act as nucleotide-exchange factors for heterotrimeric G-proteins, leading to the exchange of GDP for GTP on the G-protein α subunit, which then dissociates from both the receptor and the $\beta\gamma$ heterodimer [5]. Both GTP-bound Gα and G$\beta\gamma$ modulate an ever-increasing number of downstream effectors, such as enzymes, transporters and ion channels (reviewed in [6]). Hence, the guanine nucleotide-sensitive signal transduction machinery represents a highly complex network that also cross-talks with other systems, such as growth factor-linked signalling systems [7–10]. At the G-protein level, signalling is controlled in part by the GTPase activity of Gα, which is stimulated by G-protein-coupled receptors. This is not, however, the only process controlling the α and $\beta\gamma$ states of activation. For instance, the RGS proteins (regulators of G-protein signalling) increase the GTPase activity of the Gα subunit, resulting in the accumulation of GDP-Gα and its re-association with the free $\beta\gamma$ subunit [11]. Moreover, signalling via $\beta\gamma$ is also attenuated by calmodulin and phosducin, which bind to the $\beta\gamma$ subunit directly and translocate it to the cytosol, thus preventing its association with Gα or its effectors [12,13].

We have recently proposed that the $\beta\gamma$ heterodimer can be directly regulated through an ADP-/de-ADP-ribosylation cycle [14]. Mono-ADP-ribosylation is a regulatory post-translational modification in which the ADP-ribose moiety of NAD^+ is enzymically transferred to various proteins [15,16]. The identification and cloning of mono-ADP-ribosyltransferases and ADP-ribosylarginine hydrolase from eukaryotic cells [15,17] support the existence of a regulatory ADP-ribosylation cycle resembling the one that controls nitrogen fixation in the photosynthetic bacterium *Rhodospirillum rubrum*, where the dinitrogenase reductase activity is controlled by the reversible ADP-ribosylation of the nitrogenase enzyme complex [18].

We have previously demonstrated that the G-protein β subunit is a substrate for endogenous mono-ADP-ribosylation *in vivo* and suggested that this modification might modulate $\beta\gamma$ activity [14]. In the present study, to address this possibility, we examine the effect of ADP-ribosylation on $\beta\gamma$-subunit-mediated activation of key effectors. The free $\beta\gamma$ heterodimer can directly activate several effectors, including adenylate cyclase, phosphoinositide 3-kinase (PI 3-kinase), phospholipase C-β (PLC-β) and voltage-gated Ca^{2+} and K^+ channels [6,19,20]. By the regulation of effectors such as PI 3-kinase-γ and PLC-β2, the $\beta\gamma$ heterodimer participates in the control of many cellular functions.

The class I PI 3-kinases, which can phosphorylate phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, are the best characterized PI 3-kinases: they are key signalling enzymes, with crucial roles in many biological func-

Abbreviations used: ART1, arginine-specific ADP-ribosyltransferase; PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; CHO, Chinese-hamster ovary; MIBG, meta-iodobenzylguanidine.

[1] Present address: Institute of Pharmacology and Toxicology, University of Marburg, Karl-von-Frisch-Str. 1, 35033 Marburg, Germany.

[2] To whom correspondence should be addressed (e-mail corda@dcbo.negrisud.it or mdigirolamo@dcbo.negrisud.it).

© 2002 Biochemical Society

tions, including proliferation, differentiation, cytoskeletal organization, membrane trafficking and apoptosis [21,22]. Direct activation of the class IB PI 3-kinase p110γ catalytic subunit by the $\beta\gamma$ heterodimer has been demonstrated [19]. PLC enzymes hydrolyse the membrane lipid phosphatidylinositol 4,5-bisphosphate, simultaneously producing two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, which mediate intracellular calcium release and protein kinase C activation respectively. To date, four subfamilies of PLC are known, including four PLC-β isoenzymes activated by both the $\alpha_{q/11}$ and $\beta\gamma$ subunits, two PLC-γ isoenzymes, four PLC-δ isoforms (reviewed in [23]) and the very recently identified PLC-ϵ [24,25]. Direct activation of PLC-β isoenzymes by the $\beta\gamma$ heterodimer has been demonstrated [20].

We have examined the role that endogenous ADP-ribosylation plays in the $\beta\gamma$-subunit-mediated activation of these enzymes. This modification completely blocks the activation of both, indicating that ADP-ribosylation of the β subunit might be a general modulatory mechanism for these signalling proteins. We have also examined the mechanism by which the $\beta\gamma$ mono-ADP-ribosylation is activated and have shown that it can be controlled by G-protein-coupled receptors.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium was purchased from EurobioLabtek, Hanks balanced salt solution from Gibco, TNM-FH insect medium from Sigma, foetal bovine serum from Seromed–Biochrom and [^{32}P]NAD from Amersham. All other reagents were obtained from Sigma Chemicals at the highest purities available. GDP-αi3 was a gift from Dr V. Malhotra (University of California, CA, U.S.A.). Some of the purified bovine brain $\beta\gamma$ and the polyclonal antibody SW28 were generously supplied by Dr W. F. Simonds (National Institutes of Health, Bethesda, MD, U.S.A.). A polyclonal antibody raised against the C-terminus of the β subunit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Recombinant baculoviruses encoding GST-p101 and p110γ were a gift from R. Wetzker (Friedrich-Schiller-University, Germany).

Cell culture, plasma membrane and cytosol preparation

Chinese-hamster ovary (CHO) cells were grown in monolayers at 37 °C in 95 % air/5 % CO_2 in Dulbecco's modified Eagle's medium supplemented with 10 % foetal bovine serum, 34 μg/ml proline, 100 units/ml penicillin and 100 mg/ml streptomycin. CHO plasma membranes were prepared as described by Gettys et al. [26], with the modifications described previously [14]. Sf9 cells were grown in monolayers at 27 °C in TNM-FH insect medium supplemented with 10 % foetal bovine serum, 1 % glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 10 mg/ml gentamicin.

Purified protein preparations

Recombinant GDP-αi3 was purified to homogeneity from Sf9 cells as described previously [27] and was concentrated to 3 mg/ml in 20 mM Hepes (pH 8.0), 100 mM NaCl, 3 mM $MgCl_2$ and 1 mM EDTA. Bovine brain $\beta\gamma$ was purified as described previously [28,29] and concentrated to 2 mg/ml in 50 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol and 0.7 % CHAPS. The recombinant arginine-specific ADP-ribosyltransferase (ART1)–GST fusion protein was purified as described previously [30] and resuspended in PBS. Recombinant PI 3-kinase-γ was purified to apparent homogeneity from Sf9 cells co-infected with baculoviruses encoding GST-p101 and p110γ, as described previously [31]. Purity of all the protein samples (> 90 % pure) was verified by SDS/PAGE followed by silver staining of 200 ng of each protein. Controls for each of the individual assays (see below) were handled under the same sample incubation conditions (time of incubation, temperature, buffers and detergent).

ADP-ribosylation assay and immunoblot analysis

Samples (4 μg of CHO plasma membrane protein) were incubated at 37 °C for 60 min in 50 μl of 50 mM potassium phosphate buffer (pH 7.4) with 30 μM β-NAD and 1–2 μCi [^{32}P]NAD (specific radioactivity, 1000 Ci/mmol) as described previously [14]. In the experiments where 200 ng of purified bovine brain $\beta\gamma$ subunit was added to the sample, the association of the exogenous $\beta\gamma$ subunit with plasma membranes was evaluated by immunoblot analysis of the plasma membrane pellet and supernatant (separated by centrifugation; 15 min, 12000 ***g***), with at least 95 % of the $\beta\gamma$ subunit recovered with the membrane fraction. For extensive endogenous ADP-ribosylation of the β subunit, the incubation was performed with 1 mM β-NAD and 4.5 μCi [^{32}P]NAD for 6 h at 37 °C in the same buffer. When experiments were performed in the presence of recombinant ART1, 500 ng of $\beta\gamma$ subunit and actin were used to obtain better signal detection of both proteins. Moreover, for pertussis toxin-catalysed ADP-ribosylation of the G_i/G_o α subunits, CHO cells were incubated for 15 h with pertussis toxin (5 nM) or, as a control, with the inactive protomer B of pertussis toxin. The samples were analysed by SDS/PAGE (10 % gel), and proteins were electroblotted (4 h at 500 mA) on to nitrocellulose membranes. The filters were either exposed for approx. 12 h to Kodak X-Omat film using an intensifying screen and quantified with an Instantimager (Packard), or probed with specific primary and peroxidase-conjugated secondary antibodies using chemiluminescence (ECL®; Amersham).

PI 3-kinase-γ purification and assay

The lipid kinase activity of GST-PI 3-kinase-γ was determined as described previously [31]. The effect of the ADP-ribosylated $\beta\gamma$ subunit was evaluated as follows: 4 μg of plasma membrane protein from CHO cells that had been incubated for 6 h at 37 °C with 200 ng purified bovine brain $\beta\gamma$ subunit, without ('unmodified') or with ('modified') 1 mM β-NAD$^+$. In the presence of β-NAD$^+$, approx. 80 % of the added $\beta\gamma$ subunit was ADP-ribosylated [14]. After the incubation, CHO membranes containing either unmodified or modified $\beta\gamma$ subunit were collected by centrifugation (15 min, 12000 ***g***), suspended with 30 μl of lipid vesicles and incubated for 10 min on ice before assaying their effect on PI 3-kinase-γ activity. Assay products on dried TLC plates were quantified with an Instantimager. The stimulation of PI 3-kinase-γ obtained under our experimental conditions (i.e. incubation at 37 °C for 6 h of the membrane-associated $\beta\gamma$; see Figure 1A) was approx. 5-fold lower than that obtained using fresh $\beta\gamma$ (results not shown). The decreased activity of $\beta\gamma$ might be due to the prolonged incubation of $\beta\gamma$ or the presence of plasma membranes in the assay, or both; however, we have not investigated this aspect further. Since this level of stimulation was consistent in all our studies, we have referred to this as the appropriate control.

PLC-β2 assay

PLC-β2 activity was evaluated as described previously [32], with the exception that a crude soluble extract from baculovirus-

© 2002 Biochemical Society

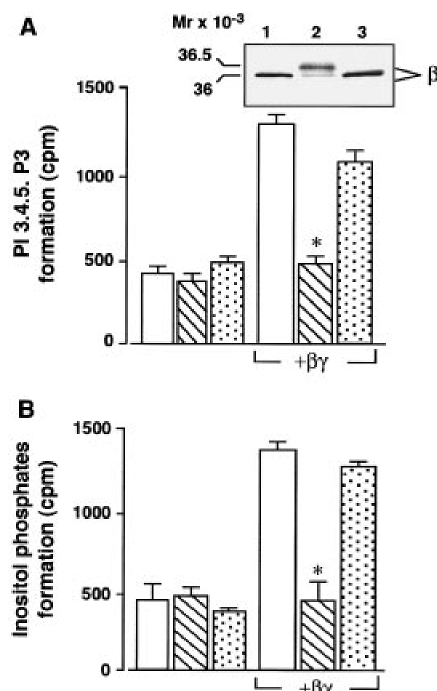

Figure 1 $\beta\gamma$-subunit-induced activation of PI 3-kinase-γ and PLC-β2 is blocked by ADP-ribosylation

PI 3-kinase-γ (**A**) and PLC-β2 (**B**) activities were evaluated as described in the Materials and methods section without or with (as indicated) 200 ng of purified bovine brain $\beta\gamma$ subunit, after incubation with either CHO plasma membranes alone (open bar), CHO plasma membranes and β-NAD (ruled bar), or CHO plasma membranes and α-NAD (dotted bar). Data are means $\pm$ S.D. for three experiments performed in duplicate ($n = 6$; **A**) and in triplicate ($n = 9$; **B**). $^{*}P < 0.01$, significantly different from the samples incubated with membranes and $\beta\gamma$ (open bar) or with membranes, $\beta\gamma$ and α-NAD (dotted bar). The inset shows an immunoblot of CHO plasma-membrane-associated purified bovine brain $\beta\gamma$ (200 ng): unmodified (lane 1), incubated with membranes and β-NAD (lane 2), or incubated with membranes and α-NAD (lane 3).

infected Sf9 cells was employed as a source of PLC-β2 [33,34]. For infection, a PLC-β2 deletion mutant lacking the C-terminal region that is required for Gαq subunit stimulation (F819-E1166) and carrying an alanine-for-serine replacement at position 2 was used. The effect of the ADP-ribosylated $\beta\gamma$ subunit was evaluated as described above for the PI 3-kinase-γ assay; after the incubation, CHO membranes containing either unmodified or modified $\beta\gamma$ subunit were collected by centrifugation (15 min, 12000 ***g***) and dispersed in the PLC assay mixture. PLC-β2 activity, as previously demonstrated for CaM-stimulated AC1 [14], was not affected by CHO membranes alone (the source of ADP-ribosyltransferase in our assays) and was only slightly reduced after prolonged incubation of $\beta\gamma$ at 37 °C, when compared with fresh $\beta\gamma$-dependent stimulation. The stimulation obtained is in the same range as that of previously reported experiments employing the same construct [33,34].

Statistical analysis

Data are expressed as means $\pm$ S.E. or as means $\pm$ S.D. Statistical analysis was performed using the Student's t test ($P < 0.05$ was considered significant).

RESULTS

Mono-ADP-ribosylation abolishes $\beta\gamma$-dependent stimulation of PI 3-kinase-γ and PLC-β2

We have demonstrated previously that the G-protein β subunit is a substrate for endogenous mono-ADP-ribosylation *in vivo* and have suggested that this modification might modulate $\beta\gamma$ activity [14]. As $\beta\gamma$ is also known to regulate several of its target molecules by direct interactions with a common effector-binding site that includes Arg-129, the target of this mono-ADP-ribosylation [14,35–38], we have now evaluated the effect of this

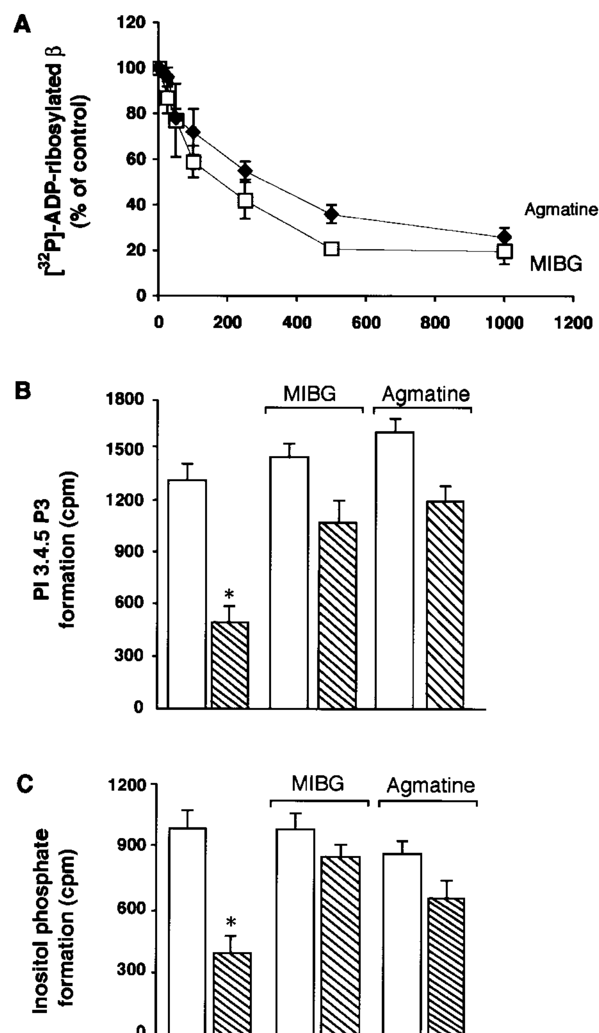

Figure 2 $\beta\gamma$-subunit-induced activation of PI 3-kinase-γ and PLC-β2 is unaffected when the ADP-ribosylation of $\beta\gamma$ is performed in the presence of inhibitors of the arginine-specific mono-ADP-ribosyltransferases

(**A**) Inhibition of β subunit ADP-ribosylation by increasing concentrations of the ART1 inhibitors MIBG and agmatine. The levels of ADP-ribosylated β subunit were measured using an Instantimager. The data represent means $\pm$ S.E. for four experiments performed in duplicate ($n = 8$). PI 3-kinase-γ (**B**) and PLC-β2 (**C**) activities were evaluated as described in the Materials and methods section: in the presence of 200 ng of either unmodified (open bars) or ADP-ribosylated with β-NAD (ruled bars) purified bovine brain $\beta\gamma$ subunits associated with CHO plasma membranes, and in the absence or presence (as indicated) of 300 μM MIBG or 300 μM agmatine. MIBG and agmatine cannot affect the PI 3-kinase-γ and PLC-β2 activities either in the absence (results not shown) or presence of the $\beta\gamma$ heterodimer (**B**, **C**; compare the open bars). The values are means $\pm$ S.E. for three experiments performed in duplicate ($n = 6$). $^{*}P < 0.01$, significantly different from the samples incubated with membranes and $\beta\gamma$ (open bar).

© 2002 Biochemical Society

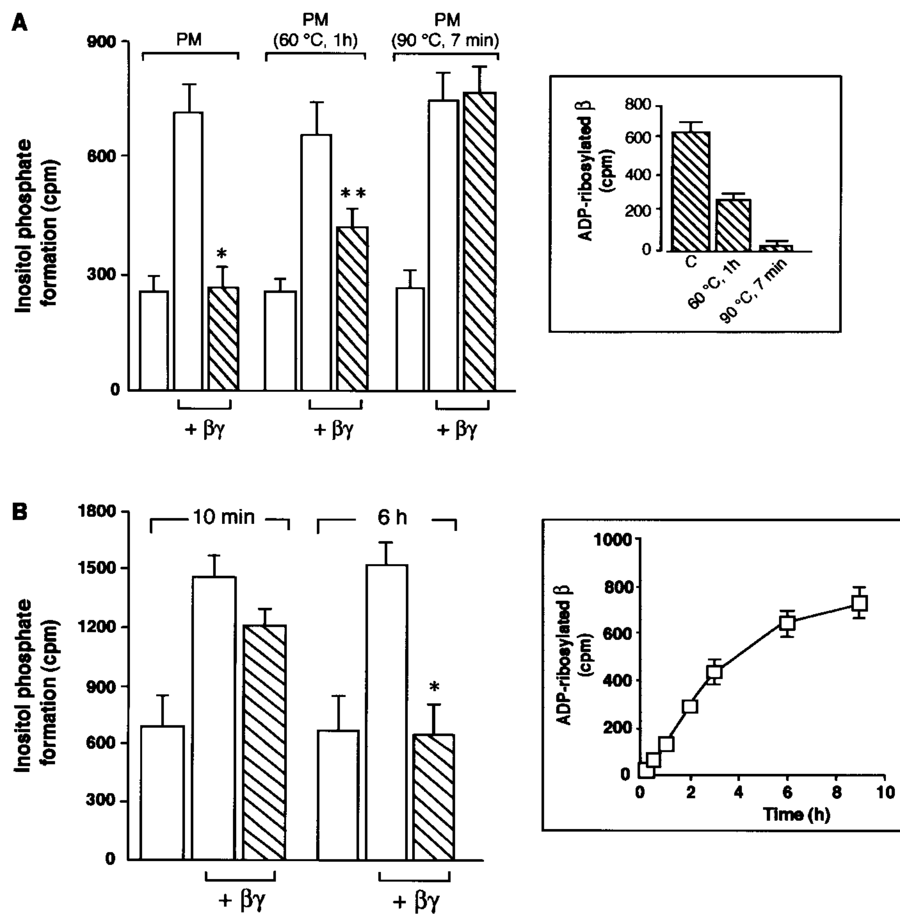

Figure 3 Inverse correlation between $\beta\gamma$-subunit-induced activation of PLC-β2 and its ADP-ribosylation

(**A**) PLC-β2 activity evaluated in the absence or presence (as indicated) of 200 ng of either unmodified (open bar) or ADP-ribosylated with β-NAD (ruled bar) purified bovine brain $\beta\gamma$ subunits associated with CHO plasma membranes (PM), which had been pretreated for 1 h at 60 °C or for 7 min at 90 °C. The loss of activity of the ADP-ribosylated $\beta\gamma$ subunit on PLC-β2 was not due to a change in association of $\beta\gamma$ with membranes, since equivalent amounts of membrane-bound β subunit were found under all the experimental conditions employed (results not shown). The data represent means ± S.E. for three experiments performed in triplicate ($n = 9$). $^*P < 0.01$ and $^{**}P < 0.05$, significantly different from the samples incubated with membranes and $\beta\gamma$ (open bar). The inset shows the levels of β subunit ADP-ribosylation when CHO plasma membranes, after pretreatment for 1 h at 60 °C or for 7 min at 90 °C, were ADP-ribosylated for 6 h at 37 °C in the presence of 200 ng of purified bovine brain $\beta\gamma$ subunits, as evaluated using an Instantimager. The data represent means ± S.E. for three experiments performed in duplicate ($n = 6$). (**B**) PLC-β2 activity evaluated in the presence or absence (as indicated) of 200 ng of either unmodified (open bar) or ADP-ribosylated for the indicated times with β-NAD (ruled bar) purified bovine brain $\beta\gamma$ subunits associated with CHO plasma membranes. The data represent means ± S.E. for three experiments performed in triplicate ($n = 9$). $^*P < 0.01$, significantly different from the samples incubated with membranes and $\beta\gamma$ (open bar). The inset shows the time dependence of β subunit ADP-ribosylation when CHO plasma membranes, containing 200 ng of purified bovine brain $\beta\gamma$ subunits, were incubated with β-NAD, as evaluated using an Instantimager. The data shown are from five experiments performed in duplicate ($n = 10$).

modification on the $\beta\gamma$-induced activation of purified PI 3-kinase-γ and PLC-β2.

PI 3-kinase-γ activity was quantified by measuring [^{3}H]phosphatidylinositol 3,4,5-trisphosphate formation catalysed by an affinity-purified PI 3-kinase-γ enzyme [31]. In agreement with previously reported results [21,22], the purified bovine brain $\beta\gamma$ subunit can activate PI 3-kinase-γ (Figure 1A, open bar), whereas the $\beta\gamma$ heterodimer previously ADP-ribosylated in the presence of β-NAD$^+$ and CHO cell membranes (inset of Figure 1, lane 2) was unable to activate PI 3-kinase-γ (Figure 1A, hatched bar). Similarly, when PLC-β2 activity was quantified by the standard method of accumulation of [^{3}H]inositol phosphates produced by an enriched preparation of soluble PLC-β2 [32–34], the stimulation seen with the purified bovine brain $\beta\gamma$ subunit (Figure 1B, open bar) was lost after prior ADP-ribosylation (Figure 1B, hatched bar). When the ADP-ribosylation assays were performed in the presence of α-NAD$^+$ (instead of β-NAD$^+$), which does not serve as a substrate for mono-ADP-ribosyltransferases (thus under conditions where ADP-ribosylation of the $\beta\gamma$ subunit does not occur; inset of Figure 1, lane 3), the $\beta\gamma$-dependent activation of both PI 3-kinase-γ and PLC-β2 was unaffected (dotted bars in Figures 1A and 1B respectively). The inset of Figure 1 shows that the loss of activity of the ADP-ribosylated $\beta\gamma$ subunit was not due to a change in association of $\beta\gamma$ with membranes, since equivalent amounts of membrane-bound β subunit were found under all the experimental conditions employed (lane 1, unmodified β; lane 2, ADP-ribosylated β; and lane 3, α-NAD$^+$-treated β; see the Materials and methods section).

In addition, when ADP-ribosylation of $\beta\gamma$ was performed in the presence of alternative, competing substrates of ART1 [300 μM meta-iodobenzylguanidine (MIBG) or 300 μM agmatine; Figure 2A], under conditions where ADP-ribosylation of the β subunit was inhibited, the $\beta\gamma$-dependent activation of PI 3-kinase-γ and PLC-β2 was unaffected (hatched bars in Figures 2B and 2C respectively). MIBG and agmatine were shown not to affect the PI 3-kinase-γ and PLC-β2 activities either

© 2002 Biochemical Society

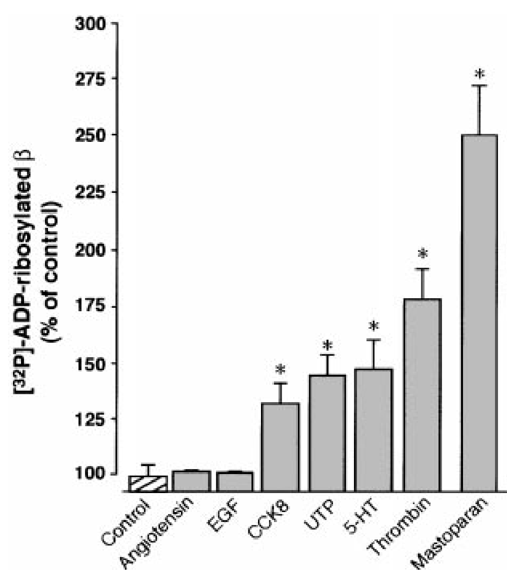

Figure 4 G-protein β subunit ADP ribosylation induced by receptor activation and mastoparan

Level of ADP-ribosylated β subunit induced by the indicated agonists and by mastoparan incubated at 37 °C for 60 min with CHO plasma membranes and [^{32}P]NAD, as measured using an Instantimager. The data represent means ± S.D. for five independent experiments performed in duplicate ($n = 10$). $^{*}P < 0.01$, significantly different from the control.

in the absence or presence of the $\beta\gamma$ heterodimer (Figures 2B and 2C, compare the open bars). When CHO plasma membranes treated either for 1 h at 60 °C or for 7 min at 90 °C to inactivate the ADP-ribosyltransferase activity (by 60 and 90 % respectively; Figure 3A, inset) were used to ADP-ribosylate the $\beta\gamma$ subunit, the $\beta\gamma$-dependent activation of PLC-β2 was reduced accordingly (Figure 3A, compare hatched bars with open bars). Finally, when the ADP-ribosylation of the $\beta\gamma$ heterodimer was performed for short time periods (i.e. 10 min each), which are insufficient to induce the modification of the β subunit (Figure 3B, inset), the $\beta\gamma$-dependent activation of PLC-β2 was not significantly affected (Figure 3B, compare hatched bars with open bars). Altogether, these results are consistent with a loss of the ability of $\beta\gamma$ to activate PI 3-kinase-γ and PLC-β2 that is selectively due to ADP-ribosylation of $\beta\gamma$ by the membrane-associated ADP-ribosyltransferase.

Agonists modulate mono-ADP-ribosylation of the $\beta\gamma$ subunit in plasma membranes

Our results demonstrate that mono-ADP-ribosylation of the β subunit abolishes the ability of the $\beta\gamma$ heterodimer to modulate downstream effectors, such as type 1 adenylate cyclase [14], PI 3-kinase-γ and PLC-β2. It is well known that the active free $\beta\gamma$ heterodimer, which directly modulates effector proteins, is generated when an activated receptor causes GDP–GTP exchange on the G-protein α subunit [5]. To evaluate whether the free $\beta\gamma$ heterodimer released upon agonist stimulation of G-protein-coupled receptors could be endogenously mono-ADP-ribosylated, we examined the level of [^{32}P]ADP-ribosylated β subunit after stimulation of CHO plasma membranes with agonists, which have specific surface receptors on these cells. CHO plasma membranes stimulated with CCK8 (cholecystokinin), UTP and 5-HT (all at 1 μM) showed a 50 % average increase in β subunit [^{32}P]ADP-ribosylation. An 80 % increase was observed after thrombin (10 units/ml) stimulation (Figure 4). In contrast, treatment with epidermal growth factor (1 μg/ml) and angiotensin II (1 μM), whose receptors are not expressed in CHO cells, did not affect β subunit [^{32}P]ADP-ribosylation. Under the same experimental conditions, a greater stimulation (ranging from 2- to 3-fold, depending on different plasma membrane preparations) of β subunit mono-ADP-ribosylation, was induced by 25 μM mastoparan (Figure 4), a tetradecapeptide known to activate heterotrimeric G-proteins by mimicking the action of activated receptors [39]. To exclude non-specific effects of mastoparan due to its amphiphilic properties, different mastoparan derivatives were used as positive and negative controls. Mastoparan 17, an inactive mastoparan analogue, did not induce β subunit mono-ADP-ribosylation, whereas mastoparan 7, the most active mastoparan derivative, caused a more pronounced β subunit mono-ADP-ribosylation (190 ± 30 % increase, as compared with mastoparan) (results not shown). Moreover, when plasma membranes obtained from CHO cells that had been incubated for 15 h with pertussis toxin to inactivate G_i/G_o proteins were analysed in an ADP-ribosylation assay, the stimulation of the β subunit mono-ADP-ribosylation by mastoparan 7 was partially blocked (35 ± 5 % decrease, as compared with the control), indicating that part of the effect of mastoparan 7 was due to the activation of pertussis toxin-sensitive heterotrimeric G-proteins (results not shown). These results indicate that the release of free $\beta\gamma$ heterodimer due to activation of G-protein-coupled receptors or mastoparan addition leads to mono-ADP-ribosylation of the β subunit, which may result in a modulation of its function. At present, the mechanisms involved in this hormone modulation are being investigated.

Mono-ADP-ribosylation of the β subunit occurs when it is in the heterodimeric conformation

Receptor activation and mastoparan addition both result in release of the active $\beta\gamma$ subunit, which appears to be the preferred substrate for modification by the endogenous ADP-ribosyltransferase. To investigate this further, we determined whether the β subunit is mono-ADP-ribosylated only as a heterodimer or also as an $\alpha\beta\gamma$ heterotrimer using either CHO plasma membranes or purified ART1 (reviewed in [15]) as a source of mono-ADP-ribosyltransferase activity. ART1 modifies the β subunit less efficiently than the endogenous membrane-associated enzymes (approx. 10-fold less; Figure 5A), but it allows a three-component reconstituted reaction (enzyme/G-protein/NAD$^+$), which can help in the understanding of the mechanism of the interaction between the α and modified $\beta\gamma$ subunits, with no interference from other membrane components. Figure 5(A) shows that the addition of GDP-αi3 (1 μg) to the assay mixture resulted in a decrease in the ADP-ribosylation level of purified bovine brain $\beta\gamma$ (0.5 μg): an average 30 % decrease was observed when the reaction was catalysed by the plasma-membrane-associated enzyme and 70 % when the reaction was catalysed by ART1. This difference may be due to other factors present in the membrane preparation that can bind GDP-αi3. We thus further analysed this phenomenon employing the purified enzyme preparation only. Figure 5(B) shows the mono-ADP-ribosylation of 0.5 μg of purified bovine brain $\beta\gamma$ by ART1 (lane 1) in the presence of increasing amounts of purified GDP-αi3 (lanes 2–4); when the same amount (0.5 μg) of α and $\beta\gamma$ subunits was present in the assay (hence, at roughly equimolar levels), a 75 % inhibition of the β subunit mono-ADP-ribosylation occurred. Moreover, 2 μg of GDP-αi3 completely abolished the $\beta\gamma$ subunit mono-ADP-ribosylation (lane 4). This GDP-αi3-dependent inhibition could be due to the titration of the free $\beta\gamma$ heterodimer or the

© 2002 Biochemical Society

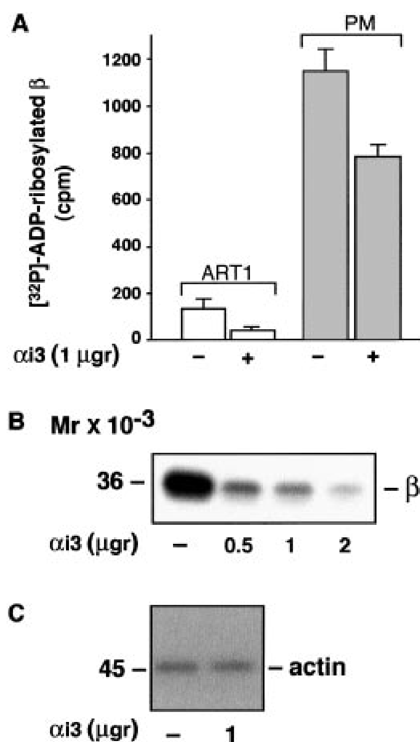

Figure 5 G-protein β subunit ADP-ribosylation is inhibited by GDP-bound αi3

(**A**) Level of ADP-ribosylated purified bovine brain $\beta\gamma$ (0.5 μg) induced by purified ART1 (3 μg) or by CHO plasma membranes (PM, 4 μg) in the absence or presence of 1 μg of GDP-bound αi3. The ADP-ribosylation assay was performed for 60 min as described above. The values are means $\pm$ S.E. for three combined experiments, each performed in duplicate ($n = 6$). (**B**) Autoradiography of β subunit (0.5 μg/sample) ADP-ribosylated for 60 min by ART1 (3 μg/sample) in the absence or presence of the indicated amounts of GDP-bound αi3. The data shown are from at least three independent experiments. A part of these data are included in (**A**). (**C**) Autoradiography of actin (0.5 μg/sample) ADP-ribosylated for 60 min by ART1 (3 μg/sample) in the absence or presence of 1 μg of GDP-bound αi3. The data shown are from at least three independent experiments.

inhibition of the mono-ADP-ribosyltransferase activity. In control experiments, GDP-αi3 had no effect on the ART1-catalysed ADP-ribosylation of actin, a well-characterized substrate of arginine-specific mono-ADP-ribosyltransferases [40] (Figure 5C). Since the addition of GDP-αi3 inhibits the mono-ADP-ribosylation of the β subunit but does not inhibit the mono-ADP-ribosylation of actin (both modified by ART1), we can conclude that this inhibition occurred by interaction with free $\beta\gamma$ heterodimer and formation of the trimeric complex.

These results thus demonstrate that the $\beta\gamma$ subunit is mono-ADP-ribosylated in its dissociated form, and they are in agreement with the demonstration that mono-ADP-ribosylation of the β subunit is enhanced both by mastoparan and activated receptors.

DISCUSSION

We recently reported the first demonstration that the G-protein β subunit is endogenously mono-ADP-ribosylated in intact cells by an arginine-specific, plasma-membrane-associated enzyme [14,16]. Moreover, we showed that the modified β subunit is de-ADP-ribosylated by a cytosolic enzyme, hence completing an ADP-/de-ADP-ribosylation cycle that may have physiological relevance [14,16]. The results presented here substantiate our proposal that this ADP-/de-ADP-ribosylation cycle is a novel cellular mechanism that can regulate the G-protein-mediated signal transduction machinery through the direct regulation of the activity of the $\beta\gamma$ subunit, which is modified when dissociated from the α subunit.

Other proteins, such as desmin, members of the integrin family, and the regulatory γ subunit of cGMP phosphodiesterase, undergo reversible mono-ADP-ribosylation [41–43]. However, most of these studies were performed *in vitro*, except in two cases where it was conclusively demonstrated that cellular proteins are indeed modified in living cells [14,44]. This is the case with GRP78/BiP, a chaperone resident in the endoplasmic reticulum, which is endogenously ADP-ribosylated in intact cells [44,45]. This modification involves oligomerization of the protein to an inactive form and is promoted by conditions depleting the endoplasmic reticulum of processable proteins [46]. More recently, mitochondrial glutamate dehydrogenase has been shown to be inactivated by cysteine mono-ADP-ribosylation in living cells [47]; however, the nature of the enzyme involved in this modification has not been clarified. Thus evidence is emerging that reversible mono-ADP-ribosylation controls the activity of signalling molecules in a way that might be analogous to the better understood system of reversible protein phosphorylation.

It is well known that the physiological processes regulated by the G-protein $\beta\gamma$ dimer are mediated by interactions between $\beta\gamma$ and several target molecules, including adenylate cyclases, PI 3-kinase, PLC-β and ion channels [5,6]. Mono-ADP-ribosylation of the β subunit occurs on Arg-129 [14], a residue located in the region of the β subunit that has been characterized as the common effector-binding surface [35–38]; thus this modification might be crucial in blocking $\beta\gamma$ activity in general. In the present study, we demonstrate that endogenous mono-ADP-ribosylation of the G-protein β subunit abolishes its ability to activate purified PI 3-kinase-γ and an enriched preparation of PLC-β2. These two effector enzymes were selected because of their well-known direct interactions with the $\beta\gamma$ subunit that take place during their G-protein-dependent modulation [19,20]. Thus, in line with this concept, the most linear interpretation of our results is that the ADP-ribose on the modified $\beta\gamma$ subunit prevents the interaction with these effectors. One cannot exclude the possibility that an indirect modulation of PI 3-kinase-γ and PLC-β2 by $\beta\gamma$ may also take place. However, this indirect mechanism would not change the original hypothesis and conclusion of these experiments, i.e. that once mono-ADP-ribosylated, the $\beta\gamma$ subunit is unable to modulate $\beta\gamma$-dependent enzymes, such as PI 3-kinase-γ and PLC-β2. Thus these results greatly strengthen the proposal that mono-ADP-ribosylation can be a general signal-termination mechanism for $\beta\gamma$ activity. It is noteworthy that, like the β subunit of trimeric G-proteins, other cellular proteins involved in signalling and cell organization, such as GRP78/BIP, integrin α7, desmin and CtBP/BARS, are substrates of mono-ADP-ribosylating enzymes [41,42,46,48–50]. In all these cases, it has been demonstrated that ADP-ribosylation leads to protein inactivation.

The $\beta\gamma$-mediated regulation of effectors requires agonist stimulation of G-protein-coupled receptors [6]. To evaluate whether the active $\beta\gamma$ heterodimer released upon agonist stimulation of G-protein-coupled receptors could be endogenously mono-ADP-ribosylated, we examined the level of the [^{32}P]ADP-ribosylated β subunit formed on acute stimulation of CHO plasma membranes with different agonists through their specific receptors. Thus we employed agonists of the G-protein-coupled P2Y2 purinergic receptor [51], the thrombin receptor [52] and the CCKA cholecystokinin receptor [53], all coupled with PLC via Gq; and of the

© 2002 Biochemical Society

serotonin (5-HT 1B-like) receptor, which is coupled via a pertussis toxin-sensitive G-protein to mitogen-activated protein kinase activation [54]. All these agonists (UTP, thrombin, CCK8 and 5-HT) induce an increase in β subunit [^{32}P]ADP-ribosylation, indicating that the active $\beta\gamma$ heterodimer released from different classes of G-proteins, such as Gq and G_i, can be a substrate for the endogenous mono-ADP-ribosyltransferase. This receptor-induced increase in β subunit [^{32}P]ADP-ribosylation might appear low (ranging from 50 to 80 %); however, this is not surprising since only a subset of G-proteins specifically coupled with the activated receptor is involved in the reaction. Indeed, non-specific activation of G-proteins by mastoparan resulted in a remarkable stimulation of the β subunit mono-ADP-ribosylation (up to 6-fold in the presence of mastoparan 7; results not shown). Mastoparan is a well-known receptor mimic: it activates trimeric G-proteins by interacting with the C-terminal domain of the α subunit [39,55], thus inducing the release of active $\beta\gamma$ heterodimer. Pretreatment of CHO cells with pertussis toxin partially decreased the effect of mastoparan 7, with approx. 60 % of the mastoparan 7-induced stimulation of β subunit mono-ADP-ribosylation being pertussis toxin-insensitive. Our results thus indicate that mastoparan 7 may activate β subunit mono-ADP-ribosylation in CHO membranes by both activating heterotrimeric G-proteins and indirectly through an interaction with the ADP-ribosyltransferase or with other plasma membrane, pertussis toxin-insensitive factors affecting the ADP-ribosyltransferase activity; this is thus similar to that demonstrated for the nucleoside diphosphate kinase [56].

Altogether, these results demonstrate that the receptor-released $\beta\gamma$ heterodimer is modified and support the hypothesis that cell activation due to stimulation of G-protein-coupled receptors could lead to the inhibition of $\beta\gamma$ activity through its mono-ADP-ribosylation. In agreement with this, when the $\beta\gamma$ heterodimer was inhibited by incorporation into an inactive trimeric complex by preincubation with equimolar amounts of purified GDP-αi3, its mono-ADP-ribosylation was markedly reduced (Figure 5), further indicating that the β subunit can be modified only when it is in its free, heterodimeric, active conformation.

It should also be considered that aside from the release of the $\beta\gamma$ heterodimer, the mechanism of receptor-induced ADP-ribosylation could involve a direct activation of the cellular transferase. Recent reports have in fact indicated that G-protein-coupled receptors interact with a variety of cellular molecules in addition to heterotrimeric G-proteins, including the adaptor molecule arrestin, primarily involved in targeting these receptors for endocytosis, and the small G-proteins Rho and Arf [57]. Thus the transferase could also be among the direct interactors of these receptors, although no data are available at present to substantiate this hypothesis. The mechanisms involved in this hormone modulation are presently being investigated.

Another possibility is that mono-ADP-ribosylation represents a regulatory mechanism that can block the $\beta\gamma$ complex when its activity is not required, for instance when heterotrimeric G-proteins are activated (by hormonal stimulation), but only the α subunit activity is needed. In other words, receptor activation would result in a short-lived increase in the free $\beta\gamma$ heterodimer, which can be subject to subsequent inactivation by ADP-ribosylation.

Thus consideration of any of the mechanisms proposed above leads to the conclusion that the β subunit mono-ADP-ribosylation cycle can have a regulatory role in modulation of G-protein-mediated signalling. Full elucidation of the physiological control of the $\beta\gamma$ ADP-ribosylation will be possible only after the molecular characterization of the specific enzyme(s) involved in this reaction.

We thank Dr A. Luini for helpful discussions, Dr M. Vaughan for a critical reading of the manuscript, Dr R. Wetzker for providing the PI 3-kinase baculoviruses and Dr W. F. Simond for the gift of β subunit antisera. This work was supported in part by the Italian Association for Cancer Research (Milano, Italy), Telethon (Italy) grant no. E.841 and the Italian National Research Council (CNR, Rome, Italy) Progetto Finalizzato 'Biotecnologie' (ctr. no. 01.00027.PF49). R.L. was supported by a fellowship from the Italian Federation for Cancer Research (Milano, Italy).

REFERENCES

1 Schwindinger, W. F. and Robishaw, J. D. (2001) Heterotrimeric G-protein $\beta\gamma$-dimers in growth and differentiation. Oncogene **20**, 1653–1660

2 Turner, P. R., Mefford, S., Christakos, S. and Nissenson, R. A. (2000) Apoptosis mediated by activation of the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein (PTHrP). Mol. Endocrinol. **14**, 241–254

3 Jin, T., Zhang, N., Long, Y., Parent, C. A. and Devreotes, P. N. (2000) Localization of the G protein $\beta\gamma$ complex in living cells during chemotaxis. Science **287**, 1034–1036

4 Pinxteren, J. A., O'Sullivan, A. J., Tatham, P. E. and Gomperts, B. D. (1998) Regulation of exocytosis from rat peritoneal mast cells by G protein $\beta\gamma$-subunits. EMBO J. **17**, 6210–6218

5 Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E. and Sigler, P. B. (1996) The 2.0 Å crystal structure of a heterotrimeric G protein. Nature (London) **379**, 311–319

6 Clapham, D. E. and Neer, E. J. (1997) G protein $\beta\gamma$ subunits. Annu. Rev. Pharmacol. Toxicol. **37**, 167–203

7 Schmidt, P., Holsboer, F. and Spengler, D. (2001) β(2)-Adrenergic receptors potentiate glucocorticoid receptor transactivation via G protein $\beta\gamma$-subunits and the phosphoinositide 3-kinase pathway. Mol. Endocrinol. **15**, 553–564

8 Hobson, J. P., Rosenfeldt, H. M., Barak, L. S., Olivera, A., Poulton, S., Caron, M. G., Milstien, S. and Spiegel, S. (2001) Role of the sphingosine-1-phosphate receptor EDG-1 in PDGF-induced cell motility. Science **291**, 1800–1803

9 Saito, Y. and Berk, B. C. (2001) Transactivation: a novel signaling pathway from angiotensin II to tyrosine kinase receptors. J. Mol. Cell Cardiol. **33**, 3–7

10 Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) Signal characteristics of G protein-transactivated EGF receptor. EMBO J. **16**, 7032–7044

11 Iyengar, R. (1997) There are GAPS and there are GAPS. Science **275**, 42–43

12 Danner, S. and Lohse, M. J. (1996) Phosducin is a ubiquitous G-protein regulator. Proc. Natl. Acad. Sci. U.S.A. **93**, 10145–10150

13 Liu, M., Yu, B., Nakanishi, O., Wieland, T. and Simon, M. (1997) The Ca^{2+}-dependent binding of calmodulin to an N-terminal motif of the heterotrimeric G protein $\beta\gamma$ subunit. J. Biol. Chem. **272**, 18801–18807

14 Lupi, R., Corda, D. and Di Girolamo, M. (2000) Endogenous ADP-ribosylation of the G protein β subunit prevents the inhibition of type 1 adenylyl cyclase. J. Biol. Chem. **275**, 9418–9424

15 Okazaki, I. J. and Moss, J. (1999) Characterization of glycosylphosphatidylinositiol-anchored, secreted, and intracellular vertebrate mono-ADP-ribosyltransferases. Annu. Rev. Nutr. **19**, 485–509

16 Di Girolamo, M. and Corda, D. (2002) Mono-ADP-ribosylation of heterotrimeric G proteins. In Handbook of Cell Signalling (Haley, ed.), Academic Press, San Diego, in the press

17 Moss, J., Stanley, S. J., Nightingale, M. S., Murtagh, Jr, J. J., Monaco, L., Mishima, K., Chen, H. C., Williamson, K. C. and Tsai, S. C. (1992) Molecular and immunological characterization of ADP-ribosylarginine hydrolases. J. Biol. Chem. **267**, 10481–10488

18 Ludden, P. W. (1994) Reversible ADP-ribosylation as a mechanism of enzyme regulation in procaryotes. Mol. Cell Biochem. **138**, 123–129

19 Leopoldt, D., Hanck, T., Exner, T., Maier, U., Wetzker, R. and Nurnberg, B. (1998) G$\beta\gamma$ stimulates phosphoinositide 3-kinase-γ by direct interaction with two domains of the catalytic p110 subunit. J. Biol. Chem. **273**, 7024–7029

20 Camps, M., Hou, C., Sidiropoulos, D., Stock, J. B., Jakobs, K. H. and Gierschik, P. (1992) Stimulation of phospholipase C by guanine-nucleotide-binding protein $\beta\gamma$ subunits. Eur. J. Biochem. **206**, 821–831

21 Rameh, L. E. and Cantley, L. C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. J. Biol. Chem. **274**, 8347–8350

22 Vanhaesebroeck, B. and Waterfield, M. D. (1999) Signaling by distinct classes of phosphoinositide 3-kinases. Exp. Cell Res. **253**, 239–254

23 Morris, A. J. and Scarlata, S. (1997) Regulation of effectors by G-protein α- and $\beta\gamma$-subunits. Recent insights from studies of the phospholipase C-β isoenzymes. Biochem. Pharmacol. **54**, 429–435

24 Lopez, I., Mak, E. C., Ding, J., Hamm, H. E. and Lomasney, J. W. (2001) A novel bifunctional phospholipase C that is regulated by Gα 12 and stimulates the Ras/mitogen-activated protein kinase pathway. J. Biol. Chem. **276**, 2758–2765

© 2002 Biochemical Society

25 Song, C., Hu, C. D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohge, M., Wu, D., Satoh, T. and Kataoka, T. (2001) Regulation of a novel human phospholipase C, PLC-ϵ, through membrane targeting by Ras. J. Biol. Chem. **276**, 2752–2757
26 Gettys, T. W., Sheriff-Carter, K., Moomaw, J., Taylor, I. L. and Raymond, J. R. (1994) Characterization and use of crude α-subunit preparations for quantitative immunoblotting of G proteins. Anal. Biochem. **220**, 82–91
27 Graber, S. G., Figler, R. A. and Garrison, J. C. (1992) Expression and purification of functional G protein α subunits using a baculovirus expression system. J. Biol. Chem. **267**, 1271–1278
28 Sternweis, P. C. and Robishaw, J. D. (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J. Biol. Chem. **259**, 13806–13813
29 Casey, P. J., Graziano, M. P. and Gilman, A. G. (1989) G protein $\beta\gamma$ subunits from bovine brain and retina: equivalent catalytic support of ADP-ribosylation of α subunits by pertussis toxin but differential interactions with Gs α. Biochemistry **28**, 611–616
30 Kim, H. J., Okazaki, I. J., Takada, T. and Moss, J. (1997) An 18-kDa domain of a glycosylphosphatidylinositol-linked NAD:arginine ADP-ribosyltransferase possesses NAD glycohydrolase activity. J. Biol. Chem. **272**, 8918–8923
31 Maier, U., Babich, A. and Nurnberg, B. (1999) Roles of non-catalytic subunits in G$\beta\gamma$-induced activation of class I phosphoinositide 3-kinase isoforms β and γ. J. Biol. Chem. **274**, 29311–29317
32 Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C. and Gilman, A. G. (1993) Purification from Sf9 cells and characterization of recombinant Gq α and G11 α. Activation of purified phospholipase C isozymes by G α subunits. J. Biol. Chem. **268**, 14367–14375
33 Dietrich, A., Meister, M., Brazil, D., Camps, M. and Gierschik, P. (1994) Stimulation of phospholipase C-β 2 by recombinant guanine-nucleotide-binding protein $\beta\gamma$ dimers produced in a baculovirus/insect cell expression system. Requirement of γ-subunit isoprenylation for stimulation of phospholipase C. Eur. J. Biochem. **219**, 171–178
34 Dietrich, A., Brazil, D., Jensen, O. N., Meister, M., Schrader, M., Moomaw, J. F., Mann, M., Illenberger, D. and Gierschik, P. (1996) Isoprenylation of the G protein γ subunit is both necessary and sufficient for $\beta\gamma$ dimer-mediated stimulation of phospholipase C. Biochemistry **35**, 15174–15182
35 Yan, K. and Gautam, N. (1996) A domain on the G protein β subunit interacts with both adenylyl cyclase 2 and the muscarinic atrial potassium channel. J. Biol. Chem. **271**, 17597–17600
36 Yan, K. and Gautam, N. (1997) Structural determinants for interaction with three different effectors on the G protein γ subunit. J. Biol. Chem. **272**, 2056–2059
37 Chen, Y., Weng, G., Li, J., Harry, A., Pieroni, J., Dingus, J., Hildebrandt, J. D., Guarnieri, F., Weinstein, H. and Iyengar, R. (1997) A surface on the G protein β-subunit involved in interactions with adenylyl cyclases. Proc. Natl. Acad. Sci. U.S.A. **94**, 2711–2714
38 Weng, G., Li, J., Dingus, J., Hildebrandt, J. D., Weinstein, H. and Iyengar, R. (1996) Gβ subunit interacts with a peptide encoding region 956–982 of adenylyl cyclase 2. Cross-linking of the peptide to free G$\beta\gamma$ but not the heterotrimer. J. Biol. Chem. **271**, 26445–26448
39 Ross, E. M. and Higashijima, T. (1994) Regulation of G-protein activation by mastoparans and other cationic peptides. Methods Enzymol. **237**, 26–37
40 Terashima, M., Yamamori, C. and Shimoyama, M. (1995) ADP-ribosylation of Arg-28 and Arg-206 on the actin molecule by chicken arginine-specific ADP-ribosyltransferase. Eur. J. Biochem. **231**, 242–249

Received 25 April 2002/15 July 2002; accepted 30 July 2002
Published as BJ Immediate Publication 30 July 2002, DOI 10.1042/BJ20020660

41 Huang, H. Y., Graves, D. J., Robson, R. M. and Huiatt, T. W. (1993) ADP-ribosylation of the intermediate filament protein desmin and inhibition of desmin assembly *in vitro* by muscle ADP-ribosyltransferase. Biochem. Biophys. Res. Commun. **197**, 570–577
42 Zolkiewska, A. and Moss, J. (1993) Integrin α 7 as substrate for a glycosylphosphatidylinositol-anchored ADP-ribosyltransferase on the surface of skeletal muscle cells. J. Biol. Chem. **268**, 25273–25276
43 Bondarenko, V. A., Desai, M., Dua, S., Yamazaki, M., Amin, R. H., Yousif, K. K., Kinumi, T., Ohashi, M., Komori, N., Matsumoto, H. et al. (1997) Residues within the polycationic region of cGMP phosphodiesterase γ subunit crucial for the interaction with transducin α subunit. Identification by endogenous ADP-ribosylation and site-directed mutagenesis. J. Biol. Chem. **272**, 15856–15864
44 Leno, G. H. and Ledford, B. E. (1989) ADP-ribosylation of the 78-kDa glucose-regulated protein during nutritional stress. Eur. J. Biochem. **186**, 205–211
45 Ledford, B. E. and Leno, G. H. (1994) ADP-ribosylation of the molecular chaperone GRP78/BiP. Mol. Cell Biochem. **138**, 141–148
46 Laitusis, A. L., Brostrom, M. A. and Brostrom, C. O. (1999) The dynamic role of GRP78/BiP in the coordination of mRNA translation with protein processing. J. Biol. Chem. **274**, 486–493
47 Herrero-Yraola, A., Bakhit, S. M., Franke, P., Weise, C., Schweiger, M., Jorcke, D. and Ziegler, M. (2001) Regulation of glutamate dehydrogenase by reversible ADP-ribosylation in mitochondria. EMBO J. **20**, 2404–2412
48 De Matteis, M. A., Di Girolamo, M., Colanzi, A., Pallas, M., Di Tullio, G., McDonald, L. J., Moss, J., Santini, G., Bannykh, S., Corda, D. et al. (1994) Stimulation of endogenous ADP-ribosylation by Brefeldin A. Proc. Natl. Acad. Sci. U.S.A. **91**, 1114–1118
49 Di Girolamo, M., Silletta, M. G., De Matteis, M. A., Braca, A., Colanzi, A., Pawlak, D., Rasenick, M. M., Luini, A. and Corda, D. (1995) Evidence that the 50-kDa substrate of brefeldin A-dependent ADP-ribosylation binds GTP and is modulated by the G-protein $\beta\gamma$ subunit complex. Proc. Natl. Acad. Sci. U.S.A. **92**, 7065–7069
50 Weigert, R., Silletta, M. G., Spano, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polishchuk, E. V., Salmona, M. et al. (1999) CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. Nature (London) **402**, 429–433
51 Iredale, P. A. and Hill, S. J. (1993) Increases in intracellular calcium via activation of an endogenous P2-purinoceptor in cultured CHO-K1 cells. Br. J. Pharmacol. **110**, 1305–1310
52 Dickenson, J. M. and Hill, S. J. (1997) Transfected adenosine A1 receptor-mediated modulation of thrombin-stimulated phospholipase C and phospholipase A2 activity in CHO cells. Eur. J. Pharmacol. **321**, 77–86
53 Dickenson, J. M. and Hill, S. J. (1998) Involvement of G-protein $\beta\gamma$ subunits in coupling the adenosine A1 receptor to phospholipase C in transfected CHO cells. Eur. J. Pharmacol. **355**, 85–93
54 George, S. E., Bungay, P. J. and Naylor, L. H. (1997) Functional coupling of endogenous serotonin (5-HT1B) and calcitonin (C1a) receptors in CHO cells to a cyclic AMP-responsive luciferase reporter gene. J. Neurochem. **69**, 1278–1285
55 Mousli, M., Bueb, J. L., Bronner, C., Rouot, B. and Landry, Y. (1990) G protein activation: a receptor-independent mode of action for cationic amphiphilic neuropeptides and venom peptides. Trends Pharmacol. Sci. **11**, 358–362
56 Klinker, J. F. and Seifert, R. (1995) Synthetic lipopeptides activate nucleoside diphosphate kinase in HL-60 membranes. Biochem. Biophys. Res. Commun. **209**, 575–581
57 Marinissen, M. J. and Gutkind, J. S. (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol. Sci. **22**, 368–376

© 2002 Biochemical Society