Gβ5γ2 Is a Highly Selective Activator of Phospholipid-dependent Enzymes*

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In this study, Gβ specificity in the regulation of Gβγ-sensitive phosphoinositide 3-kinases (PI3Ks) and phospholipase Cβ (PLCβ) isoforms was examined. Recombinant mammalian Gβ1γ2 complexes purified from SF9 membranes stimulated PI3K lipid kinase activity with similar potency (10–30 nM) and efficacy, whereas transducin Gβγ was less potent. Functionally active Gβ5γ2 dimers were purified from SF9 cell membranes following coexpression of Gβ5 and Gγ2-His. This preparation as well as Gβ1γ2-His supported pertussis toxin-mediated ADP-ribosylation of Gαi1. Gβ1γ2-His stimulated PI3K lipid and protein kinase activities at nanomolar concentrations, whereas Gβ5γ2-His had no effect. Accordingly, Gβ1γ2-His, but not Gβ5γ2-His, significantly stimulated the lipid kinase activity of PI3Kβ in the presence or absence of tyrosine-phosphorylated peptides derived from the p85-binding domain of the p110α subunit of PI3Kβ. Conversely, both preparations were able to stimulate PLCβ2 and PLCβ1. However, Gβ1γ2-His, but not Gβ5γ2-His, activated PLCβ2. Experimental evidence suggests that the mechanism of Gβ5-dependent effector selectivity may differ between PI3K and PLCβ. In conclusion, these data indicate that Gβ subunits are able to discriminate among effectors independently of Gα due to selective protein-protein interaction.

G-protein-coupled receptors are part of a major signal recognition system in mammalian cells that perceives a vast variety of external physical and chemical entities (1). These stimuli provoke cellular responses, thereby regulating almost any cellular function, including secretion, cell movement, growth, and differentiation. Accordingly, hundreds of ligands interact with >1000 of these heptahelical receptor proteins (2, 3). In turn, ligand-occupied G-protein-coupled receptors couple to dozens of heterotrimeric G-protein combinations, which transduce the incoming signal into the cell (4, 5). Both GTP-bound Gα and Gβγ modulate an ever increasing number of enzymes, transporters, or ion channels (6). On the G-protein level, signaling is controlled by the GTPase activity of Gα; stimulated by G-protein-coupled receptors; and fine-tuned by modulatory elements, including RGS (regulators of G-protein signaling) proteins (7). Hence, the guanine nucleotide-sensitive signal transduction machinery represents a highly complex network that also cross-talks to other systems such as growth factor-linked signaling systems. Different levels of finicky organization are required for a synchronized function. These include spatial and temporal expression of constituents as well as their specific and selective interaction. Accordingly, studies in living cells employing antisense RNA have demonstrated a remarkable specificity in the interaction between receptors and G-proteins (8). However, signaling specificity in whole cells appears to be significantly higher as compared with isolated and reconstituted components, which points to additional factors such as cell compartmentation or cell-specific protein expression patterns (9, 10). Nevertheless, selective protein-protein interactions obviously represent the first step in signaling specificity.

Gα heterogeneity correlates with specificity in G-protein-effector coupling. This is reflected by the subdivision of Gα isoforms into four subfamilies that activate (Gαi/o) or inhibit (Gαo) adenylyl cyclases, stimulate phospholipase Cβ isoforms (Gαq), or regulate Rho proteins (Gα12/13). A corresponding structure-function relationship is missing for Gβγ dimers despite the existence of 7 Gβ and 12 Gγ isoforms, which are grouped into two and three subfamilies, respectively, and probably form hundreds of distinct combinations. Only Tβγ4 (Gβ4γ1) differs, being consistently less potent in its ability to regulate effectors as compared with most other Gβγ combinations (6). This difference is thought to be due to the C_{LS} farnesyl moiety of Gγ1, whereas more potent isoforms are C_{LT} geranyleranylated. Hence, the lipophilic modification is assumed as an important criterion determining the potency by which Gβγ complexes modulate effectors. Furthermore, Gβ1γ4 isoforms show a high amino acid sequence identity from 79 to 90%, which may in part explain the observed low level of effector specificity. Nevertheless, major contact sites with effectors are located on the Gβ subunit, which represent the structural basis to establish selective protein-protein interactions (11).

Gβ5, the youngest member of the Gβ subunit family, appears to be an exception. Its amino acid sequence exhibits only 53% identity to other Gβ isoforms with an insertion of additional 13

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1 The abbreviations used are: Tβγ, transducin Gβγ; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; P4,4,5-P2, phosphatidylinositol 4,5-bisphosphate; P3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; NTA, nitrotriacetic acid; PAGE, polyacrylamide gel electrophoresis; Gβγ2 Iso, bovine brain Gβγ; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
amino acids at the N terminus (12). This region is thought to be important for dimerization with Gγ as well as effector coupling, recommending Gβδ as the candidate isoform to examine signaling specificity. In fact, a rather unique coupling pattern of Gβδ has been reported. It not only binds to Gγ, but also dimerizes with RGS-6, -7, -9, and -11 proteins through interaction with a Gγ-like domain (13-17). Reconstitution experiments indicated that Gβδ complexed to Gγ may selectively interact with Goα-coupled receptors through specific association of Gβδ with Goα proteins (18, 19). Moreover, Gβγ transfection studies have suggested differences in the regulation of downstream signaling pathways such as mitogen-activated protein kinase and c-Jun N-terminal kinase by Gβγ as compared with Gβγ (20). However, the molecular mechanisms for this unusual behavior remain unclear.

To examine the possibility of a so far unrecognized Gβδ-dependent specificity in effector regulation, we tested purified preparations of Gβδ complexed to Gγ for their ability to regulate PI3K and PLCβ isoforms. Heterodimeric Gβδγ was functionally active, as it supported pertussis toxin-catalyzed ADP-ribosylation of purified Goa1. However, Gβδγ did not stimulate the lipid and protein kinase activities of PI3Ks. As a consequence, Gβδγ also failed to activate voltage-operated calcium channels in rat portal vein myocytes. Surprisingly, Gβδγ exhibited a remarkable specificity within different PLCβ isoforms. It potently and efficiently stimulated PLCβ2, and it stimulated PLCβ1, but not PLCβ3, with similar potency but less efficacy, suggesting that Gβ subunits are able to discriminate among highly related isoforms.

**EXPERIMENTAL PROCEDURES**

**Recombinant PI3Ks and PLCβ Isoforms**—Construction of recombinant baculoviruses for expression of PI3K subunits and PLCβ isoforms has been described (21-24). PLCβ δ, a deletion mutant of human PLCβ2 lacking a C-terminal region necessary for stimulation by Goα, is indistinguishable from wild-type PLCβ2 in terms of its interaction with PI-4,5-P2. Ca2+; and Gβγ dimers (22). The cDNA of human PLCβ2 cloned into the EcoRI site of a pBluescript vector was kindly provided by M. van Asseldonk (Department of Human Genetics, University Hospital Nijmegen, Nijmegen, The Netherlands) (25). The construct was digested with EcoRI and ligated into this site in the pVL1393 transfer vector. Recombinant baculoviruses were produced as described previously. Expression and isolation of PI3K and PLCβ isoforms were carried out according to published protocols (22, 24).

**Gβδ Complexes and Phosphoryl Peptides**—Purification of native Gβδ complexes and Tγβδ from membranes isolated from bovine brains and retinal rod outer segments, respectively, have been described elsewhere (26, 27). Baculoviruses encoding recombinant Gβγ isoforms and Gγγ were generated and characterized as detailed previously (28, 29). Membrane-associated recombinant Gβγγ complexes were purified by combined affinity and subunit exchange chromatography, followed by anion-exchange chromatography on Mono Q columns according to published protocols (30). Recombinant Gβγγ complexes were obtained by affinity chromatography on a Ni2+-NTA column as outlined below (QIAGEN, Hilden, Germany). Phosphate-buffered saline-washed cells (multiplicity of infection of 1 virus/subunit/cell) were resuspended in ice-cold buffer A (110 mM NaCl, 3 mM MgCl2, 0.1 mM EDTA, 50 mM HEPES (pH 8.0), 10 mM β-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 0.2 mM Pefabloc<sup>®</sup> SC (Roche Molecular Biochemicals, Mannheim, Germany)). Cells were disrupted by N-cavitation (30 min at 4 °C, 25 bar) or by forcing the cell suspension through a 22-gauge needle (five times) and subsequently through a 26-gauge needle (10 times). The lysate was centrifuged at 800 × g for 2 min to remove nuclei and intact cells. Membranes were recovered at 80,000 × g for 30 min and subsequently extracted for 1 h with 0.9% sodium cholate and 0.5% Lubrol for isolation of recombinant Gβγγ or recombinant Gβδγγ, respectively. The cleared extracts (100,000 × g, 1 h) were diluted five times with buffer B (20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM MgCl2, 0.5% Lubrol, 10 mM β-mercaptoethanol, and 25 mM imidazole) and incubated with Ni2+-NTA-agarose (1 ml of slurry/7.5 × 10<sup>7</sup> infected cells) pre-washed with buffer B for 1 h. The mixture was loaded onto a column cartridge and extensively washed with buffer B containing 20 mM imidazole. Thereafter, bound Goα subunits were eluted with buffer C (20 mM HEPES (pH 8.0), 100 mM NaCl, 50 mM MgCl2, 0.9% sodium cholate (recombinant Gβγ2γγ) or 0.05% Lubrol (recombinant Gβδγγ), 10 mM β-mercaptoethanol, 10 μM GDP, 90 μM Al<sub>3+</sub>, and 10 μM GTPγS). Subsequently, the proteins were released from the matrix using a gradient of imidazole (20–150 mM, steps of 10 mM) in buffer D (20 mM HEPES (pH 8.0), 50 mM NaCl, 0.5% sodium cholate (recombinant Gβδγγ), or 0.37% octyl thioglucoside (recombinant Gβγ2γγ) and 10 mM β-mercaptoethanol).

Purified proteins were quantified by Coomassie Blue staining following separation on SDS-PAGE with bovine serum albumin as the standard. Integrity of preparations of Gβδγγ dimers was analyzed by rechromatography and pertussis toxin-catalyzed ADP-ribosylation of Goa (27). The tyrosine-phosphorylated peptide used in this study, CGGY(P)MDSKDES-VDY(P)VPVMILDM, was based on that of the human platelet-derived growth factor receptor (31) and kindly donated by Dr. Andreas Stein- meyer (Scherig AG, Berlin). A non-phosphorylated peptide served as a control and had no effect on PI3K enzymatic activity.

**Gel Electrophoresis, Immunoblotting, and Antibodies**—Generation and characterization of antisera against p110 and Gβ subunits (AS 398 and AS 422) were detailed elsewhere (28, 32, 33). The polyclonal anti-p110 and monoclonal anti-p85 antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Antisera specific for human PLCβ2 and PLCβ3 were kindly gift of Dr. P. J. Parker (University College, London) and described elsewhere (34). Preparations containing PLCβ2, PLCβ3, p110γ, p110γ, p85α, and Gβ and Gγ proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Eschborn, Germany). Visualization of specific antisera was performed using the ECL chemiluminescence system (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions.

**Lipid Vesicle Pull-down Assay**—Experimental conditions for determination of Gβγ and PI3K association on lipid vesicles were similar to measurement of the enzymatic activity of PI3K. In brief, 35 μl of PI-4,5-P2-containing lipid vesicles (320 μM phosphatidyethanolamine, 300 μM phosphatidylserine, 140 μM phosphatidylcholine, 30 μM sphingomyelin, and 50 μM PI-4,5-P2) in buffer E (40 mM HEPES (pH 7.4), 120 mM NaCl, 1 mM EDTA, 7 mM MgCl2, 1 mM dithiothreitol, 1 mM β-glycero-phosphate, and 0.1% bovine serum albumin) were mixed with different concentrations of Gβδ complexes or vehicles only. After incubation on ice for 10 min, equal amounts of PI3Kβ (5 μl) were added and thoroughly mixed. After an additional 10 min on ice, 10 μl of reaction buffer (buffer E containing 40 μM ATP) were added, followed by incubation for 5 min at 30°C. The mixture was put on ice and centrifuged for 2 min at 800 × g at 4 °C. The supernatant and pellet were separated and supplemented with electrophoresis sample buffer according to Laemmli (35). Thirty μl of either solution were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Semiquantitative analysis was realized by immunoblotting using specific antisera against p110β and Gγ subunits. As a control, PI3Kβ lipid kinase activities were measured. The results have been presented elsewhere (27). The sensitivity of PI3Kβ lipid kinase activity to Gβγγ, but not to Gβδγγ, was determined as detailed previously (23, 24). It was ensured that the effects of Gβγ on PI3K activity were not affected by their vehicles due to suppression of enzymatic activity by detergents.

**Protein Kinase Assay of PI3K**—This was performed as described (24) with modifications. The assay volume was 25 μl (2–3 μCi of [γ-32P]ATP/tube) and usually contained 7 mM Mg<sup>2+</sup>. Lipid vesicles were devoid of PI-4,5-P2. The reaction was stopped with 25 μl of ice-cold twice concentrated sample buffer according to Laemmli (35). Following separation on SDS-polyacrylamide gel, proteins were transferred to nitrocellulose membranes. Dried membranes were exposed to Fuji imaging plates, and autoradiographic signals were quantitated with a Fuji BAS 1500 imager (Ratayest, Straubenhardt, Germany).

**Phospholipase Cβ Activities**—These were quantified as described previously (22) with minor modifications. In brief, 5 μl of purified Gβγ were supplemented with 10 μl of soluble fraction of insect cells; infected with baculoviruses encoding PLCβ isoforms (0.3–4.0 μg of protein/sample); and incubated for 30 or 45 min at 25°C. Lipid vesicles (30 μM PI-4,5-P2; 50 mM NaCl, 5 mM MgCl2) were added and incubated for 10 min at 30°C. The reaction was stopped with 50 mM HEPES (pH 7.2), 70 mM KCl, 3 mM EDTA, 2 mM dithiothreitol, 33 μM [1HIP1,4-5-P2 (5 Ci/mol), and 536 μM phosphatidyl ethanolamide. In control experiments, we ascertained that soluble fractions from wild-type baculovirus-infected insect cells exhibited no Gβγ-sensitive PLC activity. Crucial experiments were repeated using purified PLCβδ<sub>δ</sub> (22) and showed results identical to those obtained with soluble fractions of PLCβδ<sub>δ</sub> baculovirus-infected insect cells.
Measurement of Ca2+ Currents—Isolated myocytes from rat portal vein were obtained by enzymatic dispersion as described previously (36). Cells were seeded at density of ~10^5 cells/mm² on glass slides in physiological solution and used the same day. Reconstitution experiments including voltage clamp and membrane current recordings were carried out exactly as described previously (32). Cell capacitance was determined in each cell tested, and current density was expressed as the maximal Ba2+ current amplitude per capacitance unit (pA/pF).

RESULTS AND DISCUSSION

Different Sensitivities of PI3K Isoforms to Gβγ Complexes—

The starting point of this study was the intriguing observation that in COS cells, Gbg; in contrast to Gbg; failed to signal to the mitogen-activated protein kinase and c-Jun N-terminal kinase pathways following cotransfection with Gγ2 (20). In this cell line, different PI3Ks have been identified as essential elements in the activation of mitogenic signaling cascades by G-protein-coupled receptors (37–39). Since we and others (23, 24, 49) have previously shown to associate efficiently with Gbg; complexes (Fig. 1), we now examined the coupling of different Gβγ isoforms to these lipid kinases by employing purified preparations (Fig. 1). By doing so, we readily noticed significant differences in the sensitivity of heterodimeric PI3Kβ and PI3Kγ, when stimulated in the absence of their particular non-catalytic subunit, i.e. p101 for p110γ and p85β for p110β (Fig. 2B). Furthermore, p110γ was activated by Gbg; with identical EC50 values regardless of which substrate was used, i.e. phosphatidylinositol or PI-4,5-P2 (data not shown). As expected, the presence of the p85 adapter did not affect Gbg;-induced stimulation of p110β. In its heterodimeric state, PI3Kγ produced P1-3,4,5-P3 much more efficiently when co-stimulated with phosphotyrosyl peptides resembling an intracellular p85-binding region of the platelet-derived growth factor receptor (data not shown, but see below). Taken together, these results confirm and extend our previous findings that the Gbg; complex directly interacts with the catalytic subunit of different PI3Ks and that the non-catalytic p101 subunit of PI3Kγ sensitizes p110γ for Gbg; in a PI-4,5-P2-dependent fashion (24).

Stimulatory Activity of Defined Gβγ Dimers on PI3Kγ—Since Gbg; bov purified from Gbg; proteins represents a mixture of Gβ and Gγ isoforms, we expressed defined Gβγ subsets in Sf9 cells. Earlier studies have revealed that the Gγ subunit affects the ability of the Gbg; complex to modulate effectors (40, 41). We therefore coexpressed Gbg; isoforms with Gγ2, which was previously shown to associate efficiently with Gbg; isoforms, and subsequently purified membrane-associated G-proteins from Sf9 cells by using the approach introduced by Kozasa and Gilman (30). Therefore, purified recombinant Gbg; Gbg;γ, and Gbg;γ, as well as native Tγβγ were tested for their ability to stimulate the lipid kinase activity of PI3Kγ (Fig. 3). The results demonstrated stimulatory activity of all four preparations tested, allowing several immediate conclusions. First, no marked differences in the potency between the three Gbg; isoforms complexed to Gγ2 were visible. Second, as noticed for other effector systems, Tγβγ was less potent (EC50 > 500 nM) than Gbg; combinations containing a Gγ predicted to be geranylgeranylated. Third, EC50 values of ~30 nM were found for Gbg;γ2 preparations, indicating that recombinant Gbg; dimers exhibited potencies almost indistinguishable from that of native Gbg;γ2. It should be noted that despite some reports describing Gbg;2 as an unfavorable combination (42, 43), Gbg;γ2 in this study was expressed as a Gbg;γ2 heterotrimer in Sf9 cells and was isolated as a functional active heterodimer following exposure to aluminum fluoride. In contrast, we could not express the shorter splice variant of Gbg;2. In our hands, it did not form a complex with Gbg;γ2. Purified Gbg;γ2 stimulated not only PI3Kγ, but also PLCβ2 and PLCβ3 enzymatic activities in parallel with Gbg;γ2 (Fig. 3 and data not shown). These data corroborate a recent report that Gbg;3 complexed to Gγ2 were equally effective in stimulating basal PLC activity following transfection of COS-1 cells (44),
but do not support an earlier cotransfection study in COS-7 cells in which Gbg1γ2, unlike other Gβγ dimers, was unable to stimulate inositol phosphate production and mitogen-activated protein kinase activity (45). For possible explanations of this discrepancy, it should be noted that the earlier study did not compare Gβ expression levels. Nevertheless, accumulating experimental evidence raises the possibility that Gβ3 distinguishes among Gβγ-regulated effectors. Gβ5, unlike Gβ1 and Gβ2, appeared to be almost incapable of inhibiting N-type calcium channels following microinjection of Gβγ expression plasmids into superior cervical ganglion neurons of rats (46). Instead, expression of Gβ5 led to a near doubling of the Ca2+ current density. Furthermore, isoform specificity in the interaction of Gβ6 with effectors was shown by demonstrating that the G-protein-coupled receptor kinase 2 carboxyl terminus bound Gβ6 and Gβ2, but not Gβ1, whereas a G-protein-coupled receptor kinase 3 fusion protein bound all three Gβ isoforms (47).

**Stimulatory Activity of Gβ5γ2-His and Gβ6γ2-His on PI3K Isoforms**—To study the effector coupling of Gβγ2, we applied a different strategy for its purification. In contrast to Gβ2-His, Gβ5 formed a heterotrimer with co-infected Gα1i-His and Gγ2 in SF9 cells since Gβ5γ2 specifically dissociated from this complex upon exposure to aluminum fluoride. Unfortunately, Gβ5 came off the final Mono Q column apart from Gγ2. Anion-exchange chromatography was indispensable to remove SF9 insect Gβγ, which stimulated PI3Kγ with similar potency and efficacy as Gβγ2-His. Hence, we alternatively obtained Gβ5γ2 dimers by using hexahistidine-tagged Gγ2. As a control, we purified Gβ2γ2-His, which, like Gβ5γ2-His, was able to support pertussis toxin-catalyzed ADP-ribosylation of purified bovine brain Gαi1 (Fig. 4A).

Furthermore, we confirmed the integrity of the Gβ5γ2-His preparations under assay conditions (Figs. 4 (B and C) and 6C), ruling out dissociation of the dimer during the experiment. Next, we tested both Gβ5γ2-His and Gβ6γ2-His for their ability to stimulate PI3Kγ harboring two enzymatic entities. Since evidence is accumulating that PI3Kγ may signal through both lipid and protein kinase activities (48), we tested the effect of Gβγ dimers on either enzymatic quality. Although Gβ5γ2-His was slightly less potent than the untagged counterpart, it significantly stimulated the formation of PI-3,4,5-P3 by PI3Kγ (EC50 = 30 nM) (Fig. 5A). In contrast, Gβ6γ2-His was ineffective at all concentrations tested. A small enhancement of PI-3,4,5-P3 formation at the highest Gβ6γ2-His concentration was most likely due to minute contamination by Sf9 Gβγ. Autophosphorylation of the catalytic p110 subunit of PI3Kγ was stimulated by Gβ5γ2-His with high potency and efficacy, whereas Gβ6γ2-His was again ineffective (Fig. 5B). To check whether the inability of Gβ6γ2-His to stimulate PI3Kγ was due to the failure of interaction, we examined the capability of Gβ6γ2-His to interfere with Gβγ2-His-induced stimulation of PI3Kγ lipid kinase activity. Gβ6γ2-His at concentrations up to 400 nM (>10-fold excess) had no inhibitory effect, suggesting that Gβ6γ2-His did not compete with Gβγ2-His for binding to PI3Kγ (see below).

We also examined PI3Kβ, which, unlike PI3Kγ, is widely distributed in mammalian tissues. As described above, Gβγ2-His...
and phosphotyrosyl peptides acted in a cooperative manner to activate PI3K\(\beta\), suggesting different binding sites for either stimulus (24, 49). Whereas G\(\beta\)\(1\)\(g\)2-His stimulated PI-3,4,5-P\(3\) using pertussis toxin. The reactions were stopped by addition of an equal volume of 2× concentrated electrophoresis sample buffer and subjected to SDS-PAGE, followed by Western blotting. For visualization, dried nitrocellulose membranes were autoradiographed and analyzed using a PhosphorImager. Pertussis toxin-catalyzed \(^{32}\)P incorporation into G\(\alpha\)1 was enhanced by G\(\beta\)\(1\)\(g\)2-His and G\(\beta\)5\(g\)2-His by 45- and 25-fold, respectively. B, binding of purified G\(\beta\)\(5\)\(g\)2-His to Ni\(^{2+}\)-NTA-agarose. Purified G\(\beta\)\(5\)\(g\)2-His preparations tested for stimulation of PI3K and PLC isoforms were checked for integrity by rebinding proteins to Ni\(^{2+}\)-NTA-agarose. An aliquot of G\(\beta\)5\(g\)2-His (pool) was incubated with, washed (wash), and eluted from (imidazole) Ni\(^{2+}\)-NTA-agarose as described under “Experimental Procedures.” Comparable aliquots (15 µl) of the loaded pool (1 ml) and eluted fractions (100 µl) were subjected to SDS-PAGE and visualized by Coomassie Blue staining. Recovery of eluted G\(\beta\)5\(g\)2-His was calculated to be 70–80% of the loaded pool. Similar results were obtained using G\(\beta\)\(5\)\(g\)2-His (data not shown). C, the retention of G\(\beta\)5 on Ni\(^{2+}\)-NTA-agarose is dependent on G\(\gamma\)2-His. Comparable amounts of G\(\beta\)\(5\)\(g\)2-His and G\(\beta\)\(1\)\(g\)2-His were bound to and eluted from Ni\(^{2+}\)-NTA-agarose as described for B. Eluted fractions were subjected to SDS-PAGE, followed by Western blotting to nitrocellulose membranes. Proteins were detected using G\(\beta\)5-specific antisera (AS 422).

**FIG. 4.** A, effect of G\(\beta\)\(\gamma\) on pertussis toxin-catalyzed ADP-ribosylation of G\(\alpha\)\(i\) \(\gamma\) (150 nM) purified from bovine brain was ADP-ribosylated in the absence or presence of 300 nM purified G\(\beta\)\(1\)\(g\)2-His or G\(\beta\)\(5\)\(g\)2-His using pertussis toxin. The reactions were stopped by addition of an equal volume of 2× concentrated electrophoresis sample buffer and subjected to SDS-PAGE, followed by Western blotting. For visualization, dried nitrocellulose membranes were autoradiographed and analyzed using a PhosphorImager. Pertussis toxin-catalyzed \(^{32}\)P incorporation into G\(\alpha\)1 was enhanced by G\(\beta\)\(1\)\(g\)2-His and G\(\beta\)5\(g\)2-His by 45- and 25-fold, respectively. B, binding of purified G\(\beta\)\(5\)\(g\)2-His to Ni\(^{2+}\)-NTA-agarose. Purified G\(\beta\)\(5\)\(g\)2-His preparations tested for stimulation of PI3K and PLC isoforms were checked for integrity by rebinding proteins to Ni\(^{2+}\)-NTA-agarose. An aliquot of G\(\beta\)\(5\)\(g\)2-His (pool) was incubated with, washed (wash), and eluted from (imidazole) Ni\(^{2+}\)-NTA-agarose as described under “Experimental Procedures.” Comparable aliquots (15 µl) of the loaded pool (1 ml) and eluted fractions (100 µl) were subjected to SDS-PAGE and visualized by Coomassie Blue staining. Recovery of eluted G\(\beta\)5\(g\)2-His was calculated to be 70–80% of the loaded pool. Similar results were obtained using G\(\beta\)\(5\)\(g\)2-His (data not shown). C, the retention of G\(\beta\)5 on Ni\(^{2+}\)-NTA-agarose is dependent on G\(\gamma\)2-His. Comparable amounts of G\(\beta\)\(5\)\(g\)2-His and G\(\beta\)\(1\)\(g\)2-His were bound to and eluted from Ni\(^{2+}\)-NTA-agarose as described for B. Eluted fractions were subjected to SDS-PAGE, followed by Western blotting to nitrocellulose membranes. Proteins were detected using G\(\beta\)5-specific antisera (AS 422).

**FIG. 5.** G\(\beta\)\(5\)\(g\)2-His does not stimulate PI3K\(\gamma\) enzymatic activities. A, lipid kinase activity. G\(\beta\)\(5\)\(g\)2-His (○) and G\(\beta\)\(1\)\(g\)2-His (□) were examined for their ability to stimulate the lipid kinase activity of PI3K\(\gamma\) in a concentration-dependent fashion. \(^{32}\)P-Labeled PI-3,4,5-P3 was isolated, separated, and quantified as described under “Experimental Procedures.” Shown are an autoradiograph of one representative experiment (top) and means ± S.D. from three independent experiments (bottom). B, protein kinase activity. Purified recombinant PI3K\(\gamma\) was assayed for autophosphorylation in response to increasing concentrations of either G\(\beta\)\(5\)\(g\)2-His (○) or G\(\beta\)\(1\)\(g\)2-His (□). An autoradiograph of one representative experiment is depicted on the top. Incorporation of \(^{32}\)P into the catalytic p110\(\gamma\) subunit is illustrated as -fold stimulation of basal activity (means ± S.D.) in three independent experiments (bottom).
formation in the absence (Fig. 6A) as well as presence (Fig. 6B) of phosphotyrosyl peptides, Gβ5γ2-His did not stimulate lipid kinase activity under either condition. To further support our findings on the selective interaction of Gβ isoforms with PI3K, we examined the ability of Gβ1γ2-His and Gβ5γ2-His to recruit PI3K to phospholipid vesicles (Fig. 6C). Notably, some PI3Kβ was associated with lipid vesicles even in the absence of Gβγ (Fig. 6C, pellet). However, increasing concentrations of Gβ1γ2-His significantly enhanced the association of PI3Kβ with liposomes, whereas Gβ5γ2-His failed, although both Gβ1γ2-His and Gβ5γ2-His associated equally well with lipid vesicles. Taken together, our data strongly suggest that PI3Ks represent a Gβγ-regulated effector system completely spared by Gβ5.

GB1γ2, but Not Gβ5γ2, Stimulates Calcium Channels in Smooth Muscle Cells—To study the physiological relevance of our observations, we examined smooth muscle cells from rat portal veins, which were previously shown to express both Gβ1 and Gβ5 (50). In this cell system, we recently found a novel receptor-induced Gβγ-mediated pathway for activation of L-type voltage-operated calcium channels depending on PI3K
stimulation (32). Using a combined electrophysiological and biochemical approach, we confirmed our earlier findings that stimulation of barium currents by infusion of purified Gβγ complexes into smooth muscle cells is quantitatively blocked by wortmannin pretreatment of cells (Fig. 7A). To examine the Gβγ isofrom specificity of this pathway, we infused recombinant Gβγ preparations at concentrations known to elicit barium currents. Steady-state barium currents were obtained within 2–4 min after establishment of the whole cell recording mode, as described previously (32). Whereas Gβγ showed significantly stimulated current density, Gβ showed no effect (Fig. 7B). Furthermore, co-infusion of Gβγ together with PI3K at concentrations that are ineffective when infused separately readily enhanced barium current density. However, similar experiments with Gβγ showed no stimulation of barium currents in portal vein myocytes (Fig. 7C). This led us to conclude that endogenous Gβγ present in these smooth muscle cells would not activate voltage-operated calcium channels offering signaling specificity encoded in the direct protein-protein interaction of Gβγ and its effector.

Selective Interaction of Gβγ with PLCβ Isoforms—Transfected Gβγ was reported to potently and efficiently stimulate PLCβ in COS-7 cells (12, 20, 51). Accordingly, purified preparations of Gβγ were shown to activate turkey PLC, which resembles human PLCβ by 71% amino acid identity (19). In this context, it should be emphasized that results from transfection experiments do not necessarily match those from reconstitution studies. For instance, whereas purified Gβγ clearly stimulated PLCβ (34, 52–55) no such effect was seen following co-transfection in COS cells (56). Furthermore, cotransfected Gβγ inhibited and Gβγ stimulated adenylyl cyclase II activity (57). Conversely, purified preparations of both Gβγ isoforms stimulated isolated adenylyl cyclase II, although Gβγ was at least 30 times less potent (19). An analogous situation may concern Gβγ regulation of adenylyl cyclase V (17, 58). This prompted us to examine the effects of purified Gβγ complexes on isolated preparations of recombinant mammalian Gβγ-sensitive PLCβ isoforms.

We first ascertained that PLCβΔ and PLCβ were both sensitive to nanomolar concentrations of Gβγ (Fig. 8A). Furthermore, T1/2 and Gβγ stimulated both PLC isoforms (data not shown). As expected from experiments with turkey PLC (19), Gβγ inhibited and Gβγ stimulated adenylyl cyclase II with similar efficacies (Fig. 8B). The Gβγ concentrations required for stimulation were in the same range as those reported by other groups (59), suggesting that either Gβγ preparation was correctly folded and processed. Surprisingly, although Gβγ activated PLCβ, it consistently failed to stimulate inositol formation by PLCβ at any concentration tested (Fig. 8C). This inability of Gβγ to stimulate PLCβ was not simply caused by the presence of the hexahistidine tag since Gβγ stimulated PLCβ in PLCβΔ and PLCβΔ with a similar potency and efficacy. Moreover, under our experimental conditions, we found that nanomolar concentrations of Gβγ and Gβγ exhibited pretreated without (middle) or with (right) 100 nM wortmannin (wort.) for 1 h. B, Ba2+ currents elicited by depolarization to +10 mV from a holding potential of −40 mV in the absence (control; top left) or presence of 400 nM (pipette concentration) Gβγ (top middle) or Gβγ (top right). Mean Ba2+ current densities (±S.E.) under resting conditions (control) and in the presence of Gβγ or Gβγ are shown on the bottom. C, Ba2+ current densities under resting conditions (control) or following infusion of 0.1 nM PI3K or 100 nM Gβγ or Gβγ alone or following co-infusion of 100 nM Gβγ or Gβγ together with 0.1 nM PI3K (pipette concentrations). Data are given as means ± S.E., with the number of experiments in parentheses. *, values significantly different from those obtained under control conditions (p < 0.05). pF, picofarads.
similar stimulatory effects on PLCβ activity (data not shown). Therefore, these data unequivocally indicate a qualitatively different regulation of PLCβ isoforms by Gβγ, suggesting the possibility that Gβγ exhibits a more restricted signaling specificity than other Gβ isoforms. Hence, two questions arise from our observations: what is the molecular basis for the observed specificity, and what functional impact may these findings have?

**Molecular and Functional Implications of the Results—**Much effort has been undertaken to define the structural elements of Gβ responsible for effector coupling (11, 60–63). This led to the conclusion that, in fact, different Gβγ-regulated effectors as well as Goα have multiple and overlapping binding sites on the Gβ surface. For instance, results from site-directed mutagenesis suggested that Gβ structures involved in activation of PLCβ should contain residues on the top surface and in the outer strands of blades 2, 6, and 7 of the Gβ propeller (62, 63). More interestingly, distinct mutants of Gβγ showed a contrary ability to stimulate the closely related PLCβ1 and PLCβ3 isoforms. Unfortunately, the residues examined are highly conserved among all Gβ isoforms, which hampers more detailed reflections on structure-function relationship in the Gβ regulation of PLCβ isoforms at this point.

Little, if any, is known about the PI3K-binding sites on Gβ. However, based on our previous work (23), one can assume that they should overlap with Goα or other effector-binding sites such as those for adenylyl cyclase or PLCβ. Accordingly, the predicted PI3K-interacting area of Gβγ should be significantly different in Gβγ, precluding interaction of Gβγ with this effector class. Assessing the impact of Gβ-binding sites on the effector enzymes is even more complicated since structural features responsible for interaction of PLCβ and PI3K isoforms with Gβγ are either poorly defined or completely unknown (23, 64, 65).

As already outlined, little is known about the functional role of Gβγ. Nevertheless, Gβγ regulates a restricted number of effectors as compared with other Gβ isoforms due to the fact that it either activates effectors such as PLCβ, upon direct binding or leaves out other effectors, e.g. class I PI3Ks, since it lacks interaction. In addition, effector binding without signal transfer may represent a third tempting possibility of effector modulation by Gβγ, complexed to Goγ. In this scenario, it functions as a competitor by antagonizing other Gβγ complexes that are able to both bind and transfer signals. This assumption is supported by a recent report suggesting that the Gβ surface interacting with PLCβ can be resolved into two different entities, i.e. a general binding motif and a signal transfer region (66). Indeed, we obtained data indicating different mechanisms explaining why Gβγ failed to stimulate PI3Ks and PLCβ. Gβγ did not interact with PI3Ks, but, in contrast, did interact with PLCβ since it inhibited the stimulatory activity of Gβγ on this effector (Fig. 8D). Therefore, receptor-
activated Gβγ may affect Gβγ-dependent downstream signaling in three different ways: (i) by inducing signal transfer to effectors upon binding; (ii) by lacking binding and signal transfer; or (iii) by antagonizing Gβγ signaling by binding to effectors, but without signal transfer.

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