A naturally occurring membrane-anchored Gα_s variant, XLα_s, activates phospholipase Cβ4

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Edited by Kirill Martemyanov

Extra-large stimulatory Gα (XLα_s) is a large variant of G protein α_s subunit (Gα_s) that uses an alternative promoter and thus differs from Gα_s at the first exon. XLα_s activation by G protein–coupled receptors mediates cAMP generation, similarly to Gα_s; however, Gα_s and XLα_s have been shown to have distinct cellular and physiological functions. For example, previous work suggests that XLα_s can stimulate inositol phosphate production in renal proximal tubules and thereby regulate serum phosphate levels. In this study, we show that XLα_s directly and specifically stimulates a specific isoform of phospholipase Cβ (PLCβ), PLCβ4, both in transfected cells and with purified protein components. We demonstrate that neither the ability of XLα_s to activate cAMP generation nor the canonical G protein switch II regions are required for PLCβ stimulation. Furthermore, this activation is nucleotide independent but is inhibited by Gβγ, suggesting a mechanism of activation that relies on Gβγ subunit dissociation. Surprisingly, our results indicate that enhanced membrane targeting of XLα_s relative to Gα_s confers the ability to activate PLCβ4. We also show that PLCβ4 is required for isoproterenol-induced inositol phosphate accumulation in osteocyte-like Ocy454 cells. Taken together, we demonstrate a novel mechanism for activation of phosphoinositide turnover downstream of Gα_s-coupled receptors that may have a critical role in endocrine physiology.

G protein–coupled receptors convert signals from the extracellular environment to physiological responses by activating heterotrimeric G proteins. Among four G protein subtypes, Gα_s in the GTP-bound form, stimulates adenylyl cyclase to produce cAMP, a second messenger that activates cyclase in cells (8). In an overexpression setting, XLα_s can mediate β2 adrenergic receptor–dependent activation of adenylyl cyclase (AC) in human embryonic kidney 293 (HEK293) cells (9); and it can couple to β2 adrenergic receptor and receptors for PTH, thyroid-stimulating hormone, and corticotropin-releasing factor and mediate cAMP generation as efficiently as Gα_s in a murine cell line lacking both Gα_s and XLα_s (10). PTH activates Gα_s and Gα_q/11 signaling in renal proximal tubules to regulate serum calcium and phosphate levels through phosphate reabsorption and vitamin D synthesis in vivo (11–13). Surprisingly, XLα_s deletion in mice (XLKO) did not significantly affect cAMP production but rather decreased both basal and PTH-stimulated inositol 1,4,5-trisphosphate (IP3) production in renal proximal tubules isolated from these mice (14). Expression of XLα_s in proximal tubules of XLKO mice rescued basal and PTH-stimulated IP3 production. Overexpression of XLα_s in HEK293 cells enhanced basal and both thrombin- and PTH-stimulated IP production. That changes in IP production occurred in the absence of changes in cAMP in proximal tubules and occurred downstream of thrombin, which does not stimulate cAMP production, argues that XLα_s-stimulated IP production is not downstream of cAMP. The mechanism for how XLα_s enhances IP3 production, however, is unknown since no known isoform of phospholipase C (PLC) has been shown to respond to Gα_s or XLα_s.

Generation of IP3 involves activation of PLC enzymes, of which five isoforms have been identified to respond to G protein activation (PLCβ1–4 and PLCε). PLC enzymes...
hydrolyze phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 4-phosphate (15, 16). Phosphatidylinositol 4,5-bisphosphate hydrolysis generates diacylglycerol and IP3. Diacylglycerol regulates the activity of protein kinase C, and IP3 mobilizes intracellular Ca2+, both of which initiate multiple signaling cascades to regulate a variety of cellular processes (16). All PLCβ isoforms are activated by Gαq/11 subtype (17). PLCβ2 and PLCβ3 are also activated by Gβγ subunits (18–20). PLCɛ is a downstream effector of virtually every G protein family because of either direct regulation by G protein βγ subunits (21) or via indirect activation by small GTases of the Ras superfamily (16, 22–26). Significant progress in understanding the biochemical and physiological functions of PLCβ1, PLCβ2, PLCβ3, and PLCɛ has been made by multiple laboratories including ours. However, much less is known about the PLCβ4 isofrom. PLCβ4 is highly homologous to the NorpA PLC protein that mediates the phototransduction cascade in Drosophila (27, 28). Known biological functions of PLCβ4 are limited. PLCβ4 knockout mice develop ataxia (29) and have impaired visual processing (30).

In this report, using both cell biology and biochemical approaches, we demonstrate that PLCβ4 is selectively and directly activated by XLα4 through a mechanism that differs from canonical effector activation by G protein α subunits. These results likely explain how XLα4 regulates phosphatidylinositol (PI) hydrolysis in vivo and suggest a mechanism by which Gs-coupled receptors can activate PLC in tissues that express XLα4 and PLCβ4.

Results

**XLα4 selectively activates PLCβ4 in transfected COS-7 cells**

To begin to understand the mechanistic basis for XLα4-dependent regulation of IP production, we screened several PLC isoforms for XLα4-dependent activation. COS-7 cells were cotransfected with XLα4 and different PLC complementary DNAs (cDNAs), including PLCβ2, PLCβ3, PLCβ4, and PLCɛ and measured total IP accumulation. This approach has been used extensively to identify upstream regulators of PLC enzymes (23, 31). IP accumulation increased significantly in cells expressing XLα4 and PLCβ4 but not in cells that coexpressed XLα4 with other PLC isoforms (Fig. 1B). These PLC isoforms were all activated by their canonical G protein activators (Fig. S1) in the same assay. Increasing amounts of XLα4 cDNA cotransfected with PLCβ4 led to a concentration-dependent increase in IP production (Fig. 1, C and D).

Because the only difference between XLα4 and Gαs is their first exon (Fig. 1A), we next investigated whether Gαs can
activate PLCβ4, using the similar transfection approach in COS-7 cells. The long and short variants of Gaq resulted in a small but statistically significant increase in IP accumulation (Fig. 1, E and F). cAMP activates PKA through cAMP generation and Rap through Epac, respectively. However, cotransfection of PKA or Rap with PLCβ4 did not lead to an increase in IP accumulation (Fig. S2), supporting the idea that XLαs-dependent cAMP production is not responsible for PLCβ4 activation by XLαs.

**XLa₅ activates PLCβ4 in a reconstituted enzyme assay**

To understand whether the activation of PLCβ4 by XLαs is direct or through other mediators, we partially purified XLαs and PLCβ4 to test the ability of XLαs to activate PLCβ4 in vitro with phospholipid vesicles containing PI as the substrate. Through multiple attempts to purify XLαs, we achieved a final XLαs preparation at roughly 30% purity with any attempts at further purification leading to protein aggregation (Fig. 2A). The XLαs preparation bound to GTPγS although the exact stoichiometry could not be determined because the protein was not pure (Fig. S3). In this reconstituted assay, XLαs increased PLCβ4 enzymatic activity in a concentration-dependent manner (Fig. 2B). Direct activation of PLCβ4 by Gaq was tested as a positive control (Fig. 2C). Purified Gaq did not activate PLCβ4 (Fig. 2D). PLCβ3 was not activated by purified XLαs but was activated by Gaq in the same assay. That the XLαs preparation did not activate PLCβ3 strongly indicates that PLCβ4 activation was not because of contamination of the preparation with Gaq or contamination with a copurifying phospholipase. Overall, these data support the idea that XLαs selectively and directly activates PLCβ4.

**Activation of PLCβ4 by XLαs is independent of activation state and is inhibited by Gβγ**

The ability of G protein α subunits to engage and activate their effectors is strongly enhanced in the GTP-bound activated form. Aluminum fluoride (AlF₄⁻) forms a complex with GaGDP, resulting in GaGDP·AlF₄⁻ complex that resembles the activated GaGTP. XLαs activated PLCβ4 in vitro regardless of whether AlF₄⁻ was added (Fig. 3A). This finding was further confirmed in COS-7 cells coexpressing PLCβ4 with WT XLαs or with XLαs variant (R543H) that is constitutively active with respect to activation of adenyl cyclase (7). XLαs-R543H did not further increase IP accumulation as a result of PLCβ4 activation while having similar protein expression levels as XLαs (Fig. 3, B and C). However, XLαs-mediated PLCβ4 activation in vitro was suppressed in a concentration-dependent manner by the addition of purified Gβγ subunits (Fig. 3D). Similarly, cotransfection of GB₁γ₂ also inhibited IP accumulation by XLαs-activated PLCβ4 in COS-7 cells (Fig. 3, E and F).

**Activation of PLCβ4 by XLαs does not require GTP-dependent conformational changes in switch II region**

Canonical G protein activation upon GTP binding involves a variety of conformational changes that allow for engagement of effectors. In particular, switch II region of Gaq undergoes conformational changes upon GDP·GTP exchange that

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**Figure 2. Direct and specific activation of PLCβ4 by XLαs in a reconstituted enzyme assay.** A, PLCβ4 and XLαs were purified to ~95% and ~30%, respectively. B, titration of PLCβ4 activity with XLαs, and (C) Gaq. D, recombinant XLαs stimulates PLCβ4 in a concentration-dependent manner, whereas recombinant Gaq does not increase PLCβ4 enzymatic activity. E, recombinant Gaq stimulates PLCβ3 in a concentration manner, whereas XLαs does not. Data combined from at least three independent experiments are shown as mean ± SEM. PLCβ4, phospholipase Cβ4; XLαs, extra-large stimulatory Gaq.
enhance engagement with AC to mediate its activation. Other G protein α subunits also operate through this mechanism. Loss-of-function mutations of the glycine G226 and glutamate E268 residues in Gαs, which interact with residues in switch II region (Fig. 4A), are defective in GTP-induced activation of AC (32). We made the analogous double mutant in XLαs (G568A/Q610A; designated as XLαsMut). This XLαsMut does not stimulate cAMP production on its own and showed markedly reduced ability to mediate isoproterenol (Iso)-induced cAMP generation compared with the WT XLαs (Fig. 4B). However, this mutant activated PLCβ4 to induce IP accumulation similarly to WT XLαs (Fig. 4C) and had similar protein

Figure 3. Activation of PLCβ4 by XLαs is not nucleotide state dependent but is inhibited by Gβγ. A, specific activity of PLCβ4 in the presence of different concentrations of XLαs with or without 30 μM AlCl3 and 10 mM NaF (AlF4−). B, COS-7 cells cotransfection with PLCβ4 and XLαs or GTPase-deficient XLαs (XLαs, R543H) results in higher IP accumulation. XLαs, R543H does not lead to a higher IP accumulation. ** and **** One-way ANOVA, Dunnett post test, p < 0.01, p < 0.0001, respectively. C, Western blot of XLαs, XLαsR543H shows similar protein expression, whereas PLCβ4 expression is unchanged. XLαs R543H does not lead to a higher IP accumulation. *** and **** Two-way ANOVA, Dunnett post test, p < 0.001, p < 0.0001, respectively, or in E, cellular assay, **** one-way ANOVA, Dunnett post test, p < 0.0001. F, Western blot from COS-7 cells coexpressing PLCβ4 with or without Gβ1 and Gγ2 as indicated in E. Data combined from three to four independent experiments are shown as mean ± SEM. IP, inositol phosphate; PLCβ4, phospholipase Cβ4; XLαs, extra-large stimulatory Gα.
expression levels to WT XLαs (Fig. 4D). This provides additional support to the idea that XLαs stimulates PLCβ4 in a cAMP-independent manner and shows that XLαs engagement with PLCβ4 likely requires a different structural determinant than switch II region.

Membrane localization of XLαs is required for full activation of PLCβ4

XLαs tightly localizes to the PM compared with Gaαs, in part because of the presence of two conserved palmitoylated cysteine residues C287 and C318 and a highly charged domain within the extended N terminus (7). The individual cysteine mutations did not substantially alter PM binding, but mutation of both C287 and C318 to serine significantly decreased the localization of XLαs to the PM (Fig. 5A). COS-7 cells were transiently transfected with WT XLαs, XLαs (C287S), XLαs (C318S), or XLαs (C287S, C318S) together with PLCβ4 IP accumulation measured. Substitution of either cysteine, or both cysteine residues to serine, showed significantly reduced IP accumulation compared with cells expressing WT XLαs and PLCβ4, although IP accumulation was not entirely abolished (Fig. 5B).

Structure–activity relationship studies of XLαs reveal that targeting Ga to the PM is sufficient for activation of PLCβ4

Gaαs and XLαs are nearly identical except for their N-terminal regions (Figs. 1A and 6A), yet XLαs activates PLCβ4 with significantly higher efficacy (Fig. 1, E and F). We created a series of truncation mutations in XLαs and investigated their ability to activate PLCβ4 in cells. A cDNA construct with removal of N-terminal amino acids (amino acids 2–240) beyond the proline-rich region (PRR), and prior to the palmitoylation sites (post-PRR XLαs) (7), still markedly increased IP accumulation when cotransfected with PLCβ4 in COS-7 cells (Fig. 6B). In addition, a construct that comprises exclusively the N-terminal residues (amino acids 1–381) fused to GFP (Nterm XLαs) (33) had no effect on IP accumulation when cotransfected with PLCβ4 (Fig. 6B).

We further removed the region extending from the C-terminal end of PRR to the C-terminal end of highly charged domain (amino acids 2–345 removed) and added the Lyn membrane targeting motif (GCIKSKGKDSA) (34) at its N terminus to create Lyn-QMR-XLαs. The addition of the Lyn sequence is designed to replace the XLαs membrane targeting determinants lost in this deletion construct and maintain QMR-XLαs association with the membrane. This Lyn-QMR-XLαs construct only differs from the Gaαs at its N terminus helix (in red box, Fig. 6A), which is a putative Gβγ interacting domain in XLαs (7). IP accumulation increased significantly in cells expressing Lyn-QMR-XLαs and PLCβ4 (Fig. 6C).

Since QMR-XLαs and Gaαs differed by only a short stretch of amino acids corresponding to the amino terminus of Gaαs, we examined whether addition of the Lyn targeting sequence to the amino terminus of Gaαs would enable it to activate PLCβ4. To achieve this, we inserted the Lyn motif at the N terminus of
**XLαs regulation of phospholipase Cβ**

**Figure 5. Plasma membrane localization of XLαs is important for PLCβ4 activation.** A, schematic diagram of XLαs domain structure, WT XLαs consists of an XL domain, which contains a highly charged domain (HCD), and a proline-rich region (PRR-P). Asterisks depict the two conserved cysteines in the XL domain. Immunocytochemical analysis of subcellular distribution for WT and Cys-to-Ser mutants of XLαs in HEK293 cells by using an anti-HA antibody. HEK293 cells were transiently transfected with expression constructs encoding HA-tagged WT or Cys-to-Ser mutants of XLαs (Cys-287 and Cys-318). Twenty-four hours after transfection, subcellular localizations of these XLαs mutants were investigated. The scale bar represents 5 μM. B, total IP accumulation in COS-7 cells expressing WT XLαs or Cys-to-Ser mutants of XLαs and PLCβ4. *p < 0.001 compared with PLCβ4, one-way ANOVA, Tukey post test. *** and **** p < 0.0001, respectively, compared with XLαs + PLCβ4, one-way ANOVA, Tukey post test. Western blots show expression of PLCβ4 and different XLαs constructs tested in the IP accumulation assays. Data combined from three to four independent experiments are shown as mean ± SEM. HA, hemagglutinin; HEK293, human embryonic kidney 293 cell line; IP, inositol phosphate; PLCβ4, phospholipase Cβ4; XLαs, extra-large stimulatory Ga.

**Discussion**

In this study, we present evidence that PLCβ4 is a direct effector of an amino terminally extended variant of Gaαs, XLαs. This regulation is specific to PLCβ4 relative to other PLC isoforms, revealing a novel potential mechanism for stimulation of IP production downstream of Ga-coupled receptors that is independent of cAMP. XLαs and Gaαs are both encoded by the GNAS locus. Early reports demonstrated that XLαs did not couple to adrenergic receptors in reconstituted S49ccy3 membranes lacking Gaαs (8). However, subsequent studies demonstrated that XLαs and Gaαs have similar functions in mediating receptor-dependent stimulation of cAMP production via adenyl cyclase activation (9, 10).

We demonstrate here that unlike Gaαs, XLαs activates PLCβ4, which is considered to be a canonical Gaαq/11 effector. Since both Gaαs and XLαs stimulate cAMP production, this indicates that the effect of XLαs on IP production is not because of the actions of the cAMP targets PKA and Epac in cells. Several other lines of data support a cAMP-independent mechanism including a lack of effect of PKA transfection or Epac inhibition on PLCβ4 activation (Fig. S2). Compellingly, a mutation that disables the active conformation of the switch 2 helix in XLαs (Fig. 4) abolishes its ability to stimulate cAMP production in the absence of receptor activation but does not alter its ability to support activation of PLCβ4.

Our biochemical reconstitution experiments also support a mechanism where XLαs directly activates PLCβ4 independent of CAMP. This approach as well as Cos cell cotransfection experiments have established the canonically accepted mechanisms for regulation of PLCβ isoforms by G protein subunits (20, 31, 36–39). A caveat is that the preparation of XLαs is Gaαs short (LynGaαs). Surprisingly LynGaαs activated PLCβ4 similarly to XLαs (Fig. 6D). This indicates that specific structural features of the unique XLαs N terminus are not required for PLCβ4 activation but rather the ability of XLαs to anchor strongly to the PM allows it to interact with and activate PLCβ4.

**XLαs regulation of PLCβ4 mediates Iso-dependent IP production in osteocytes**

It has been shown that ablation of XLαs in isolated proximal tubule–enriched renal cortices and osteocyte-like Ocy454 cell line represses IP3 generation (14, 35). Because PLCβ4 is activated by XLαs, we examined if PLCβ4 mediates the effect of XLαs in maintaining basal and Iso-dependent regulation of IP production in Ocy454 cells. Iso stimulation of Ocy454 cells resulted in a small but statistically significant increase in IP production (Fig. 7A). Transfection of these cells with a pool of PLCβ4-directed siRNA oligonucleotides depleted PLCβ4 protein (Fig. 7B), whereas control (scrambled) oligonucleotides did not. Ocy454 cells with depleted PLCβ4 had reduced basal and Iso-stimulated IP production compared with cells transfected with scrambled siRNA oligonucleotides. This finding supports a role for PLCβ4 in XLαs-dependent regulation of IP signaling in osteocytes.
Figure 6. Identifying the region in XLα that activates PLCβ4. Membrane-targeting intact Ga, activates PLCβ4. A, sequence alignment of XLα and the amino terminus of Ga, long (through Ga, amino acid 138. The remainder of Ga, is identical to XLα). Red asterisks denote XLα cysteine 287 and 318. Arrow marks the beginning of the post PRR-XLα, and the QMR-XLα sequence that follow immediately after Lyn membrane targeting motif (Lyn-QMR-XLα). Red box denotes the N-terminal α-helical GBγ interaction domains in XLα and Ga, B–D, COS-7 cells were transfected with indicated plasmid constructs, and total IP accumulation was assayed as described for Figure 1A; protein expression was examined by SDS-PAGE and immunoblotting. For D, above the graph
impure and leaves the possibility that a contaminating component of the preparation is activating or facilitating activation of PLCβ4 or has intrinsic PLC activity. Multiple controls strongly argue against contamination by other G proteins or PLCs including the inability of the preparation to activate PLCβ3, which is activated by both Gαs and Gβγ. In addition, the majority of the biochemical properties of XLαs in the in vitro PLC assays were recapitulated in the intact cell cotransfection assay including nucleotide-independent activation.

We observed a statistically significant small activation of PLCβ4 by Gαs in cells that was not observed in the in vitro reconstitution experiments. One possibility is that in cells there are additional regulatory mechanisms downstream of Gαs and cAMP that can alter PLCβ4 activation independently of direct activation of PLCβ4 by XLαs. An alternate possibility is that in vitro Gαs does not interact with the phosphatidylethanolamine:PI vesicle bilayer that supplies the PI substrate and supports G protein–PLC interactions, whereas XLαs is able to bind to this membrane surface allowing it to engage with PLCβ4.

Selective knockout of XLαs in mice decreased basal and PTH-dependent IP production in renal proximal tubules but, surprisingly, did not result in a decrease in cAMP production (14). IP production was enhanced in kidney proximal tubules isolated from mice with transgenic expression of XLαs and in HEK293 cells transfected with XLαs. PTH stimulates urinary phosphate excretion, which is known to be regulated, at least partly, by IP3 production. Serum phosphate levels were significantly increased in XLαs knockout mice, which could be attributed to a resistance to PTH-stimulated IP3 production. Hereofore, no known mechanisms for PLC regulation could explain these results. Regulation of PLCβ4 by XLαs provides a likely mechanistic basis for these observations in mice and other systems. PLCβ4 is expressed in the proximal convoluted tube (40) as well as Ocy454 osteocyte–like cells (Fig. 7B), in which XLαs is also expressed and mediates IP3 production.

The mechanism for XLαs-dependent regulation of PLCβ4 diverges from classical mechanisms for G protein–dependent effector activation. In the biochemical reconstitution experiments, activation was independent of nucleotide status and in cell transfection studies did not rely on the switch II region classically involved in effector engagement. This property has been observed in Gαs-dependent activation of adenyl cyclase, where purified Gαs activated adenyl cyclase in both GDP and GTP-bound states, albeit with different potencies (41). The small G protein K-ras has also been reported to interact with its effector argonaute 2 independently of its nucleotide state (42). Despite the nucleotide-independent activation of PLCβ4 by XLαs, addition of Gβγ subunits inhibited the actions of XLαs on PLCβ4 both in cells and with purified proteins. This suggests a mechanism whereby receptors could regulate XLα–PLCβ4 interactions based on receptor-dependent dissociation of XLαs from Gβγ subunits.

Our structure function analysis demonstrated that a primary determinant of XLαs-dependent of PLCβ4 is its unique mode of membrane targeting relative to Gαs. The Gαs domain at the carboxy terminus of XLαs is identical to Gαs (Figs. 1A and 6A). Surprisingly, both XLαs and a Gαs variant containing a Lyn-targeting sequence at the amino terminus activate PLCβ4 to similar extents. The Lyn PM targeting sequence Gly Cys Ile Lys Ser Lys Gly Lys Asp Ser Ala is myristoylated at Gly1 and palmitoylated at Cys2 and is enriched in positively charged amino acids that all contribute to specific PM localization (34, 43). The XLαs amino terminus is also enriched in positively charged amino acids (44). Gαs dissociates from the PM upon activation,
whereas XLαs does not (7, 45). How Gαs is anchored to the membrane may modulate its orientation at the membrane relative to its targets, with the unique amino terminus of XLαs orienting the Gαs domain such that it can engage and activate PLC. Alternatively, prolonged residency of XLαs at the PM allows for engagement with PLCβ4. The precise molecular mechanisms for how XLαs binds and activates PLCβ4, however, requires further study.

**Experimental procedures**

**Plasmid constructs and cloning**

The Gateway entry vector encoding PLCβ4 was purchased from Genecopoeia (catalog no.: GC-Y5168-CF-GS). QuikChange mutagenesis was performed to add stop codon to the ORF. Gateway pDEST10 vector was purchased from Thermo Fisher Scientific (catalog no: 11806015). Destination vector pEZYegfp was a gift from Yu-Zhu Zhang (Addgene; plasmid #18671). The complete PLCβ4 ORF was transferred from the entry vector to pEZYegfp or Gateway pDEST10 vectors using Gateway LR Clonase II Enzyme Mix (Invitrogen; catalog no.: 11791-020), following the manufacturer’s protocol, resulting in a mammalian expression vector encoding N-terminally tagged enhanced GFP PLCβ4 and a baculovirus vector encoding N-terminally tagged 6xHis PLCβ4. XLαs in pCDNA3.1 was previously described. N-terminal-hexahistidine-tagged XLαs (His6-XLαs) was synthesized by inserting sequences encoding six histidine residues after the start codon methionine of pFastBac-XLαs made in house. Truncation mutagenesis was done using Q5 Site-Directed Mutagenesis kit (NEB). The Lyn N-terminal sequence (Gly Cys Ile Lys Ser Lys Gly Lys Asp Ser—GCIKSKGKDSA) (34) was inserted at the N terminus of Gαs right after the start codon to create Lyn-Gαs construct. Lyn-QMR-XLαs was created by performing deletion of residues 2 to 345 in XLαs and then inserted the Lyn sequence between the start codon Met and Glu346.

**Protein purification**

SF9 and High Five insect cells were maintained in SF-900 II serum-free media. Bacmids and baculoviruses were made following the Bac-to-Bac baculovirus expression system protocol (Thermo Fisher Scientific).

Purification of 6xHis PLCβ4 followed previously described protocols (46). Briefly, High Five insect cells were infected with baculovirus at a density between 1.5 × 10^6 and 2 × 10^6 cells/ml at a multiplicity of infection of 1. After 48 h, cells were harvested by centrifugation, snap frozen in liquid nitrogen, and stored at −80°C. Frozen insect cell pellets expressing His6 PLCβ4 were lysed in 15 ml lysis buffer (per liter of insect cell culture) containing 20 mM Hepes, pH 8, 50 mM NaCl, 10 mM β-mercaptoethanol (β-ME), 0.1 mM EDTA, 0.1 M EGTA, 0.1 mM DTT, protease inhibitors including 133 μM PMSF, 21 μg/ml tosyl-L-lysine chloromethyl ketone and tosyl-L-phenylalanine chloromethyl ketone, 0.5 μg/ml aprotonin, 0.2 μg/ml leupeptin, 1 μg/ml pepstatin A, 42 μg/ml tosyl-L-arginine methyl ether, 10 μg/ml soybean trypsin inhibitor by subjecting the cell suspension to four cycles of thawing in a 37°C water bath and snap freezing in liquid nitrogen. The lysate was diluted with 45 ml cold lysis buffer with addition of NaCl to a final concentration of 1 M and centrifuged at 40,000 rpm using a Ti60 rotor. The supernatant was collected and diluted 5× with buffer containing 10 mM Hepes, pH 8, 10 mM β-ME, 0.1 mM EDTA, 0.1 M EGTA, 0.5% polyoxyethylene lauryl ether (C12E10), and protease inhibitors. The diluted supernatant was then centrifuged at 100,000g, and the supernatant was loaded onto a nickel–nitrilotriacetic acid column pre-equilibrated with buffer A (20 mM Hepes, pH 8, 100 mM NaCl, 10 mM β-ME, 0.1 mM EDTA, and 0.1 M EGTA). The column was washed with three column volumes (CVs) of buffer A, followed by three CVs of buffer A supplemented with 300 mM NaCl and 10 mM imidazole. The protein was eluted from the column with 3 to 10 CVs of buffer A, supplemented with 200 mM imidazole. Proteins were concentrated and loaded onto a gel filtration Superdex column equilibrated with buffer containing 20 mM Hepes, pH 8, 200 mM NaCl, 2 mM DTT, 0.1 mM EGTA, and 0.1 mM EDTA. Fractions of His6 PLCβ4 at greater than 95% purity were confirmed by SDS-PAGE and Coomassie staining, pooled, concentrated, and snap frozen in liquid nitrogen. Protein concentrations were determined by Nanodrop absorbance at 280 nm and confirmed by a bicinchoninic acid protein assay.

6xHis-XLαs was coexpressed with Gβ1γ2 in High Five cells and purified using a nickel–nitrilotriacetic acid affinity column. Briefly, High Five cells were harvested 48 h postinfection. Cell pellets were suspended in 15 ml lysis buffer (20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, 10 mM β-ME, 10 μM GDP, and protease inhibitors) and subjected to four freeze–thaw cycles with liquid nitrogen to promote cell lysis. The resulting lysate was further diluted with lysis buffer to 80 ml and centrifuged at 35,000 rpm for 1 h. Ensuing membrane pellets were resuspended in extraction buffer (20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 10 mM β-ME, 10 μM GDP, and protease inhibitors) and homogenized. Membrane proteins were extracted by adding sodium cholate to a final concentration of 1% (v/v) and isolated via centrifugation at 35,000 rpm for 45 min. The resulting supernatant was diluted 1:5 with Ni²⁺ loading buffer A (20 mM Hepes, pH 8.0, 100 mM NaCl, 0.1 mM MgCl₂, 5 mM β-ME, 40 mM imidazole, 10 μM GDP, 0.5% C₁₂E₄₀, and protease inhibitors) and loaded onto a 1 ml HisTrap HP column (Cytiva) at 0.5 ml/min. After washing with 25 ml of Ni²⁺ load buffer A to remove nonspecific impurities, the HisTrap column was warmed to room temperature and subjected to five 2 ml washes with aluminum fluoride elution buffer (20 mM Hepes, pH 8.0, 300 mM NaCl, 10 mM MgCl₂, 5 mM β-ME, 30 mM imidazole, 10 μM GDP, 0.5% C₁₂E₄₀, and protease inhibitors) and loaded onto a 1 ml HisTrap HP column (Cytiva) at 0.5 ml/min. After washing with 25 ml of Ni²⁺ load buffer A to remove nonspecific impurities, the HisTrap column was washed with buffer A containing 10 mM Hepes, pH 8.0, 100 mM NaCl, 1.25 mM MgCl₂, 5 mM β-ME, 60 mM imidazole, 10 μM GDP, 1% CHAPS, and protease inhibitors. 6xHis-XLαs was eluted with a linear imidazole gradient constructed from 60 to 500 mM imidazole. Fractions of 1 ml were collected and analyzed using SDS-PAGE on 4 to 20% Tris–glycine Mini-
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Protein gels (Bio-Rad) followed by Coomassie staining. Fractions containing significant 6xHis-XLa₅ (molecular weight ~111 kDa) identified by SDS-PAGE, Coomassie blue staining, and Western blotting were pooled and concentrated, flash-frozen in liquid nitrogen, and stored at −80 °C until use for activity assay.

**Cell culture and [³H]-IP₄ accumulation assay**

COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) at 37 °C with 5% CO₂. Reverse transfection using Lipofectamine 2000 (Thermo Fisher Scientific) was adapted from manufacturer’s protocol. A maximal amount of DNA/well/24-well plate was 450 ng at a DNA:lipofectamine 2000 ratio of 1:3. COS-7 cells in antibiotics-free DMEM supplemented with 10% FBS were mixed with the DNA:lipofectamine 2000 in a 24-well plate at 100,000 cells/well. Approximately 24 h after transfection, the media was replaced with Ham's F10 media supplemented with 10% FBS and 1% pen/strep and Western blotting were pooled and concentrated, flash-frozen in liquid nitrogen, and stored at −80 °C until use for activity assay.

**PI hydrolysis IP-One homogeneous time-resolved fluorescence assay**

PI hydrolysis was measured using a modified version of the commercially available IP-One assay (IP-One G₄ Kit; Cisbio). Assay of PLCβ activity has been described previously except conditions were modified to use PI as the substrate for compatibility with the IP-One Assay kit (48). Hen egg white phosphatidylethanolamine and soy PI (Avanti Polar Lipids) were mixed and dried under nitrogen. Lipids were resuspended in sonication buffer (50 mM Hepes, pH 7.0, 80 mM KCl, 3 mM EGTA, and 1 mM DTT) and sonicated giving a final concentration of 300 μM phosphatidylethanolamine and 750 μM PI. Assays contained 50 mM Hepes, pH 7, 80 mM KCl, 16.67 mM NaCl, 0.83 mM MgCl₂, 3 mM DTT, 1 mg/ml bovine serum albumin (BSA), 2.26 mM Ca²⁺, and varying amounts of PLCβ₄ variant proteins and/or G proteins. XLa₅ activity was also tested for intrinsic PI hydrolysis activity in the protein preparation. Protein concentrations are indicated in the figure legends. Control reactions contained the same components but lacked CaCl₂. Reactions were initiated by addition of liposomes and transferred to 37 °C for 5 min. Reactions were quenched upon addition of 5 μl quench buffer (100 mM Hepes, pH 7, 160 mM KCl, 1 mM DTT, and 210 mM EGTA), and 14 μl of each reaction was then transferred to a 384-well plate (Greiner Bio-One). For IP detection, D2-labeled IP1 (fluorescence acceptor) and anti-IP1 cryptate (fluorescence donor) were preincubated with Detection Buffer (Cisbio). 3 μl of D2-labeled IP1 and 3 μl anti-IP1 cryptate were then added to each well used in the 384-well plate. Positive assay controls contained 50 mM Hepes, pH 7, 80 mM KCl, 16.67 mM NaCl, 0.83 mM MgCl₂, 3 mM DTT, 1 mg/ml BSA, 2.26 mM Ca²⁺, D2-labeled IP1, and anti-IP1 cryptate, whereas negative assay controls contained all components except D2-labeled IP1. The plate was then incubated for one hour in the dark at room temperature, followed by centrifugation at 1000g for 1 min. Plates were read with a Versioskan LUX Multimode plate reader (Thermo Fisher Scientific) at 610 and 665 nm. IP1 was quantified using a standard curve and data reduction protocol for normalization (Cisbio). Data were plotted, and statistics were performed using GraphPad Prism 7.0a (GraphPad Software, Inc).

**SDS-PAGE and immunoblotting**

Gel electrophoresis and Western blotting were performed as previously described (46). In brief, after transfer, the membrane was incubated with Tris-buffered saline buffer supplemented with 0.1% Tween-20 (TBS-T) and 5% BSA at room temperature for 30 min on a shaker, then probed with primary antibodies (anti-PLCβ₄ [Sigma–Aldrich; catalog no.: HPA007951], anti-Gα [Sigma–Aldrich; catalog no.: 06-237], anti-Gβ [in house], anti-GAPDH [Invitrogen; catalog no.: MA5-15738]) diluted 1:1000 in TBS buffer supplemented with 0.1% Tween-20 and 3% BSA at 4 °C overnight. The membrane was washed with TBS-T four times and probed with secondary antibody (goat anti-rabbit immunoglobulin G, DyLight 800 [Invitrogen; catalog no.: SA535571]) at room temperature for 1 h. After another four washes with TBS-T, immunoreactive proteins were visualized using Li-Cor Odyssey CLx and analyzed using Image Studio Lite software (Li-Cor).

**Immunocytochemistry**

Cells were grown and transfected in 8-well chamber slides with cover (Nunc Lab-TekII; catalog no.: 154534). Cells were...
washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 20 min. Cells were permeabilized and blocked with 0.1% saponin and 0.5% BSA in PBS for 1 h. Cells were incubated with a rabbit antihemagglutinin antibody (Abcam; catalog no.: ab137838) and then incubated with Alexa Flour 568-conjugated anti-rabbit immunoglobulin G (Invitrogen; catalog no.: A11036). The immunoreactivity was visualized and analyzed by using a spinning disc confocal fluorescent microscope at 100x.

**Quantification of cAMP generation**

pGloSensor-22F cAMP Plasmid construct (Promega) was a gift from Dr Manojkumar Puthenveedu (University of Michigan). The GNAS knockout HEK293T cell line as gift from Kirill Martemyanov (University of Florida, Scripps) (49) was maintained in DMEM supplemented with 10% FBS, glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin in an incubator at 37 °C in an atmosphere of 5% CO2 and 95% O2. Cells were transiently cotransfected with PTH1R, pGloSensor-22F, and different Gαs, XLαs, or chimera plasmid constructs in tissue culture–treated solid white 96-well plate (Costar; catalog no.: 3917). cAMP assays were performed 24 h post-transfection. Cells were equilibrated with Leibovitz’s media (Gibco) containing 150 μg/ml p-luciferin potassium salt (GoldBio) for 1 h in 37 °C incubator. After equilibration, luminescence was read before and after treating cells with varying concentration of Iso (MP Biomedicals; catalog no.: 151368) Iso using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific).

**Data availability**

All data are included in the article, but primary data files are available upon request to Alan Smrcka.

**Supporting information**—This article contains supporting information.

**Acknowledgments**—We thank Dr Gregory Tall for sending us the Gαs and Gαi proteins and Dr Kirill Martemyanov (University of Florida, Scripps) for the GNAS knockout HEK293T cell line. We thank Dr Manojkumar Puthenveedu (University of Michigan) for the XLαs proteins and Dr Kirill Martemyanov (University of Florida, Scripps) (49) was a gift from Dr Manojkumar Puthenveedu (University of Michigan). All data are included in the article, but primary data files are available upon request to Alan Smrcka.

**Author contributions**—H. T. N. P., M. B., and A. V. S. conceptualization; A. V. S. methodology; A. V. S. validation; H. T. N. P. formal analysis; H. T. N. P., J. L., and S. A. investigation; Q. H. and M. B. resources; H. T. N. P. writing—original draft; M. B. and A. V. S. writing—review & editing; A. V. S. project administration; A. V. S. funding acquisition.

**Funding and additional information**—This work was supported by National Institutes of Health grants R35GM127303 (to A. V. S.) and R01DK121776 and R01DK073911 (to M. B.).

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.
XLaα, regulation of phospholipase Cβ


