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Preactivation Permits Subsequent Stimulation of Phospholipase C by G_i-Coupled Receptors

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ABSTRACT

In the complex signal transduction networks involving G protein-coupled receptors there are numerous examples where G_i-linked receptors augment G_q-dependent signals. The mechanistic basis of such occurrences is thought to entail signal convergence at phospholipase Cβ (PLCβ) via the G protein $\beta\gamma$-dimers. Herein, we explored the possibility that augmentation by $\beta\gamma$-dimers requires preactivation of PLCβ. COS-7 cells were transiently cotransfected with cDNAs encoding various combinations of receptors and G protein subunits. The G_i-coupled δ- and κ-opioid receptors could not stimulate PLCβ unless they were coexpressed with Gα_{16}. The opioid-induced response was dose-dependent and partially inhibited by pertussis toxin or coexpression with transducin, indicating the involvement of $\beta\gamma$-subunits released from the G_i proteins. When PLCβ was preactivated by constitutively active mutants of Gα_{16}, Gα_q, or Gα_{14}, opioids enhanced the activity by 80 to 300% and such responses were mostly pertussis toxin-sensitive. The opioid-induced enhancement was dose-dependent and could not be blocked by staurosporin, a protein kinase C inhibitor. Other G_i-coupled receptors that were ineffective on their own also acquired the ability to stimulate PLCβ in the presence of a constitutively active mutant of Gα_q. Coactivation of endogenous or exogenous G_q-coupled receptors with the δ-opioid receptor produced strong stimulations of PLCβ and such responses could be partially blocked by pertussis toxin. These results show that enhancement of G_q-dependent signals by G_i-coupled receptors requires activated PLCβ and is mediated via the $\beta\gamma$-dimer.

In the nervous system, different extracellular signals are often required to coordinate complex neuronal activities such as neurotransmission and cognition. The multitude of extracellular signals is usually detected by a variety of cell surface receptors that use distinct yet overlapping signal transduction mechanisms. The ability to integrate and process incoming signals is an important characteristic of neurons. The superfamily of G protein-coupled receptors (GPCRs) constitutes a large array of cell surface detectors for neurotransmitters, hormones, lipids, pheromones, and photons. Multiple GPCRs are often coexpressed in any particular cell type, where they regulate the levels of intracellular second messengers independently, in synergism, or by antagonism. Of the two most widely studied effectors of GPCRs, adenylyl cyclase and phospholipase C (PLC), intricate regulatory mechanisms for the former have been discerned.

The mechanism by which signals generated from different GPCRs become integrated inside the cell is best exemplified by the type 2 adenylyl cyclase. Type 2 adenylyl cyclase can be stimulated by the G protein $\beta\gamma$-subunits only when it is already preactivated by either Gα_s (Federman et al., 1992) or protein kinase C-mediated phosphorylation (Tsu and Wong, 1996). Hence, the $\beta\gamma$-subunits released on the activation of G_i-linked receptors can enhance the activity of type 2 adenylyl cyclase only if G_s- or G_q-linked receptors are simultaneously activated. This unique property of type 2 adenylyl cyclase allows it to integrate and process signals from various GPCRs, perhaps providing a temporal distinction of the different inputs (Lustig et al., 1993a).

Equally important for coordinating cellular functions is the regulation of PLC that generates diacylglycerol and inositol phosphate $(IP)_3$, leading to the activation of protein kinase C and calcium mobilization. Many GPCRs stimulate PLCβ-isozymes through coupling to G proteins belonging to the G_q subfamily (Rhee and Bae, 1997). The regulation of PLCβ-isozymes by GPCRs bears some similarity to those of adenylyl cyclase. For instance, PLCβ can be stimulated by the α-subunits of all G_q subfamily members as well as by the $\beta\gamma$-dimers (Smrcka and Sternweis, 1993; Nakamura et al., 1995). The β2 and β3 isoforms of PLC are especially responsive to stimulation by $\beta\gamma$-subunits. However, most G_i-coupled

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ABBREVIATIONS: GPCR, G protein-coupled receptor; PLCβ, phospholipase Cβ; IP, inositol phosphate; PTX, pertussis toxin; fMLP, formyl peptide; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; DOR, δ-opioid receptor; DPDPE, [D-Pen^{2,5}]enkephalin; ORL, opioid receptor-like; KOR, κ-opioid receptor.

receptors are incapable of activating PLCβ despite their ability to generate free βγ-subunits. In many biological systems such as the smooth muscles and cultured astrocytes, although activation of G_i-coupled receptors alone has no effect, it augments G_q-mediated responses (for review, see Selbie and Hill, 1998). The augmentation produced by the stimulation of G_i-coupled receptors is presumably mediated by the βγ-dimers (Biber et al., 1997). These observations suggest that the βγ-subunits released on the activation of G_i are insufficient to stimulate PLCβ, and other signals or conditions may be required. A distinct possibility is that PLCβ can integrate signals from G_i-, G_s-, and G_q-linked receptors in a manner akin to the one used by the type 2 adenylyl cyclase. In the course of examining the coupling of opioid receptors to G_{16} (Lee et al., 1998), we noticed that although the opioid-induced response was mediated via G_{16}, it was partially sensitive to pertussis toxin (PTX) treatment. In this report, we describe our efforts to decipher the molecular mechansim behind such PTX sensitivity. Our results suggest that when PLCβ is preactivated by the α-subunits of G_q, G_{14}, or G_{16}, it becomes responsive to stimulation by βγ-dimers. Such a precondition by which G_i-linked receptors can stimulate PLCβ may have important mechanistic implications on signal processing by PLCβ.

Materials and Methods

Reagents. cDNAs encoding the formyl peptide (fMLP) and δ-opioid receptors were kindly provided by F. Boulay (LBIO/Laboratoire, France) and C. Evans (UCLA), respectively. The bombesin and κ-opioid receptors were gifts from J. Battey (National Institute of Neurological Disorders and Stroke) and G. Bell (University of Chicago, IL), respectively. The cDNA encoding the α-subunit of G_{L1} (the bovine homolog of G_{14}; henceforth referred to as G_{14} for generality) was generously provided by Dr. T. Nukada (Tokyo Institute of Psychiatry, Japan). The origin and construction of other cDNAs have been described elsewhere (Wong et al., 1992; Lee et al., 1998). PTX and plasmid purification columns were purchased from List Biological Laboratories (Campbell, CA) and Qiagen (Hilden, Germany), respectively. COS-7 cells were obtained from the American Type Culture Collection (ATCC CRL-1651). [^{3}H]Myo-inositol was obtained from DuPont-NEN (Boston, MA). Receptor agonists and staurosporin were purchased from Research Biochemicals (Natick, MA). Antisera against $G\alpha_{q/11}$ (3A-180) and $G\alpha_{14}$ (3A-195) were purchased from Gramsch Laboratories (Schwabhausen, Germany). Antiserum G51820 against $G\alpha_{t1}$ was from Transduction Laboratories (Lexington, KY). Cell culture reagents were obtained from Life Technologies (Grand Island, NY) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of $G\alpha_{16}$Q212L and $G\alpha_{14}$R179C Mutants. Polymerase chain reactions (PCRs) were used to generate the two mutants. The cDNAs subjected to site-directed mutagenesis were subcloned into pcDNAI (Invitrogen, San Diego, CA), which contained T7 and SP6 promoter sequences as flanking priming regions. Human $G\alpha_{16}$ and bovine $G\alpha_{14}$ were used to generate $G\alpha_{16}$Q212L ($G\alpha_{16}$QL) and $G\alpha_{14}$R179C ($G\alpha_{14}$RC). Primers encoding the desired mutations were listed below with the mismatch nucleotides underlined: *16-QL/S*: GACGTCGGAGGCCTGAAGTCAGAGCGT; *16-QL/AS*: ACGCTCTGACTTCAGGCCTCCGACGTC; *14-RC/S*: GTGCTCCGTGTCTGCGTGCCCACCACT; *14-RC/AS*: AGTGGTGGGCACGCAGACACGGAGCAC. Two overlapping fragments that contained the mutation in their overlapping region were amplified separately with thermal cycling at 94°C (1 min)/50°C (1 min)/72°C (1 min) for 30 cycles with Robocycler 40 from Stratagene (La Jolla, CA). The PCR products were annealed together and the full-length fragments were made with the flanking primers. Extension time was increased to 1.5 min/cycle. Full-length $G\alpha_{16}$QL was ligated into *Eco*RV-cut pcDNAI, whereas $G\alpha_{14}$RC was subcloned into pcDNAI as a *Pst*I/*Xba*I cassette. DNA sequences of the mutants were checked by dideoxynucleotide sequencing method with Sequenase V2.0 kit.

Transient Transfection and IP Assay. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (v/v), 50 U/ml penicillin, and 50 μg/ml streptomycin, and grown at 37°C in an environment of 5% CO_2. Cells were seeded in 12-well plates at a density of $\sim 1 \times 10^5$ cells/ml and were transfected with the appropriate cDNAs 24 h later by means of the DEAE-dextran method (Wong, 1994). One day later, the transfected cells were labeled with [^{3}H]myo-inositol (2.5 μCi/ml) in inositol-free DMEM (0.75 ml/well) containing 5% fetal calf serum (v/v) for 18 to 24 h. Where necessary, PTX (100 ng/ml) was added together with the radiolabel. Labeled cells were rinsed with 2 ml of assay medium (20 mM HEPES-buffered DMEM with 20 mM LiCl) followed by incubation at 37°C for 1 h in 1 ml of assay medium with the indicated drugs. The reaction was terminated by aspiration and addition of 0.75 ml of 20 mM formic acid. IP production was estimated by determining the ratio of [^{3}H]IP to [^{3}H]inositol plus [^{3}H]IP as described previously (Tsu et al., 1995b).

Preparation of Plasma Membranes and Immunodetection of Gα-Subunits. COS-7 cells were grown on 150-mm dishes to 70 to 80% confluence and transfected as described for 12-well plates with proper adjustments to the volumes and amounts of the reagents used. Transfected cells were harvested 48 h later in PBS (Ca^{2+} and Mg^{2+} free) containing 10 mM EDTA. Cells were resuspended in lysis buffer (50 mM Tris-HCl containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 1 mM EGTA, 5 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.4) and lysed by one cycle of freeze-thawing followed by 10 passages through a 27-gauge needle. After removal of nuclei by centrifugation, membranes were collected, washed, and resuspended in lysis buffer. Protein concentrations were determined with the Bio-Rad protein assay kit. For each sample, 50 μg of membrane proteins was separated on a 12.5% polyacrylamide SDS gel and electrophorectically transferred to polyvinylidene difluoride membranes. Localization of protein markers on the polyvinylidene difluoride membrane was by Ponceau S staining. Antigen-antibody complexes were visualized by chemiluminescence with the enhanced chemiluminescence kit from Amersham (Arlington Heights, IL).

Data Analysis. The IP levels were interpreted as the ratios of the counts per minute of [^{3}H]IP fractions to those of the total labeled inositol fractions and expressed as the ratio of [^{3}H]IP over total [^{3}H]inositols. Absolute values for IP accumulations varied between experiments, but variability within a given experiment was in general <10%. Data shown in the figures are means ± S.D. of triplicates within one single experiment. At least three independent experiments yielded similar results. Bonferroni *t* test with 95% confidence was adopted to verify the significance between different treatment groups within the experiments.

Results

$G\alpha_{16}$-Mediated Stimulation of PLCβ by G_i-Coupled Receptors Is Partially Sensitive to PTX Treatment. It has recently been shown that the δ-opioid receptor (DOR) can efficiently stimulate the formation of IP in COS-7 cells when it is coexpressed with $G\alpha_{16}$ (Lee et al., 1998). As a member of the G_q subfamily, $G\alpha_{16}$ lacks the ADP ribosylation site and is resistant to modification by PTX. $G\alpha_{16}$-mediated stimulation of PLCβ should thus be PTX-insensitive. Surprisingly, when we examined the ability of DOR to stimulate PLCβ via $G\alpha_{16}$ in transiently transfected COS-7 cells, the agonist-induced response was partially reduced by PTX. In the absence of PTX treatment, the δ-selective agonist [D-Pen^{2,5}]enkephalin (DPDPE) stimulated IP formation by 9-fold (Fig. 1). Pretreat-

ment of transfected COS-7 cells with PTX suppressed the $G\alpha_{16}$-mediated response by 55%. Three other G_i-coupled receptors that are incapable of activating PLCβ in the absence of $G\alpha_{16}$ also were examined. COS-7 cells were cotransfected with cDNAs encoding $G\alpha_{16}$ and the fMLP, opioid receptor-like (ORL_1), or A_1 adenosine receptor. Like DOR, the ability of these G_i-coupled receptors to stimulate PLCβ via $G\alpha_{16}$ was attenuated by PTX (Fig. 1). Receptor-selective agonists induced 6- to 8-fold stimulation of PLCβ activity, but these $G\alpha_{16}$-mediated responses were partially sensitive to PTX treatment. In COS-7 cells coexpressing the κ-opioid receptor (KOR) and $G\alpha_{16}$, U50,488 (a κ-selective agonist) stimulated IP accumulation in a dose-dependent manner (Fig. 2A). Again, PTX treatment reduced the U50,488-induced IP formation by ~60% and raised the EC_{50} of U50,488 for $G\alpha_{16}$-mediated stimulation of PLCβ from ~60 nM to ~200 nM (Fig. 2A). A similar shift in EC_{50} values also was observed with DOR (J.W.M.L. and Y.H.W., unpublished data) and it may reflect the efficiency of coupling solely to the transfected $G\alpha_{16}$. The potency with which PTX affects the opioid-induced stimulation of PLCβ and inhibition of adenylyl cyclase was approximately the same. The EC_{50} of PTX in suppressing the $G\alpha_{16}$-mediated stimulation of PLCβ was 0.3 ng/ml, whereas PTX blocked the opioid-induced inhibition of adenylyl cyclase with an EC_{50} of 0.5 ng/ml (data not shown).

Involvement of $\beta\gamma$-Subunits. The PTX sensitivity of the DPDPE response suggests the involvement of G_i proteins. Activation of G_i proteins by DOR will invariably lead to the dissociation of the α- and $\beta\gamma$-subunits. Because none of the $G\alpha_i$-subunits possess the ability to directly activate PLCβ, they are unlikely to stimulate the formation of IP. In contrast, the $\beta\gamma$-dimer is known to regulate a host of effectors, including different isoforms of PLCβ (for review, see Clapham and Neer, 1997). To test if $\beta\gamma$-subunits are involved in the $G\alpha_{16}$-dependent stimulation of PLCβ by DOR, we attempted to block the agonist-induced response with a $\beta\gamma$-scavenger, the α-subunit of transducin ($G\alpha_{t1}$). When $G\alpha_{t1}$ was transiently coexpressed with DOR and $G\alpha_{16}$, the DPDPE-induced response was suppressed by 60% (Fig. 2B). The extent of suppression by $G\alpha_{t1}$ was similar to that produced by PTX. Increasing the concentration of $G\alpha_{t1}$ cDNA used in the transfections from 0.25 μg/ml to 0.75 μg/ml did not further attenuate the DPDPE-induced response. The expression of $G\alpha_{t1}$ was confirmed by immunodetection with a $G\alpha_t$-specific antiserum (Fig. 2B). In our heterologous expression system, overexpression of $G\alpha_{t1}$ did not affect the expression level of $G\alpha_{16}$ (Fig. 2B). Coexpression of another $\beta\gamma$-scavenger, the carboxyl fragment of β-adrenergic receptor kinase ($\beta ARK_{495-690}$), with DOR and $G\alpha_{16}$ also suppressed the DPDPE-induced response by ~40% (data not shown). Such experiments implicate the involvement of $\beta\gamma$-subunits.

Constitutively Active Gα-Mutants Permit G_i-Linked Receptors to Stimulate PLCβ. Because DOR is incapable of stimulating endogenous PLCβ in the absence of $G\alpha_{16}$ (Lee et al., 1998), somehow the expression of $G\alpha_{16}$ allowed the endogenous PTX-sensitive G proteins to participate in the activation of PLCβ. Interestingly, mechanisms exist for per-

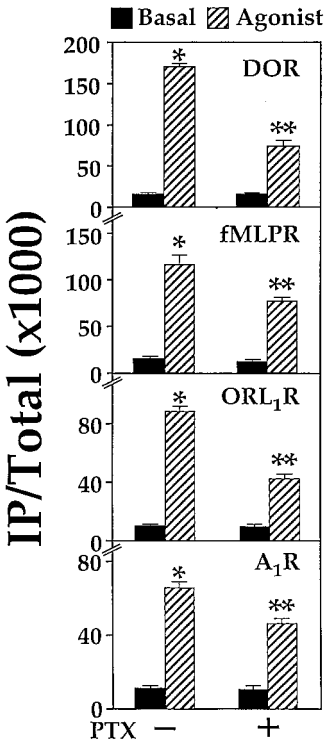

Fig. 1. $G\alpha_{16}$-mediated stimulation of PLCβ by G_i-coupled receptors is PTX-sensitive. COS-7 cells were cotransfected with cDNAs encoding $G\alpha_{16}$ and a G_i-coupled receptor: DOR, fMLPR, ORL_1R, or A_1R (each at 0.25 μg/ml). After 24 h, the cells were labeled overnight with 2.5 μCi/ml [^{3}H]myo-inositol with or without 100 ng/ml PTX as indicated. IP production was assayed in the absence or presence of an agonist: 100 nM DPDPE, 200 nM fMLP, 100 nM nociceptin, or 10 μM (+)-N^6-(2-phenylisopropyl)-adenosine. *, agonist significantly increased IP accumulation over basal values. **, PTX significantly reduced the agonist-induced response; $n = 3$, Bonferroni t test, $P < .05$.

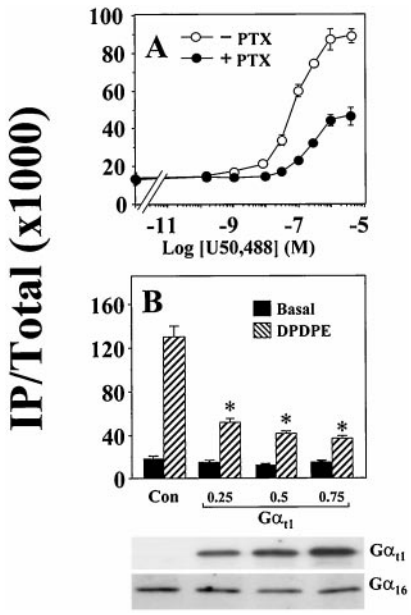

Fig. 2. Permissive stimulation of PLCβ by opioid receptors through $G\alpha_{16}$ is agonist dose-dependent and can be inhibited by $G\alpha_{t1}$. A, COS-7 cells were transfected and labeled as in (A) but with KOR instead of the other receptors. IP production increased dose-dependently with increasing concentrations of the KOR agonist U50,488 (0–3 μM). B, COS-7 cells were cotransfected with the DOR and $G\alpha_{16}$ cDNAs (both at 0.25 μg/ml) only or with varying concentrations of $G\alpha_{t1}$ (0.25 to 0.75 μg/ml). Cells were assayed for IP production in the absence or presence of 100 nM DPDPE. *, all concentrations of $G\alpha_{t1}$ significantly reduced the DPDPE-induced IP accumulation; $n = 3$, Bonferroni t test, $P < .05$. Bottom, expression of $G\alpha_{t1}$ and $G\alpha_{16}$ in the transfected cells as determined by immunodetection with antisera G51820 and 3A-180, respectively.

missive activation of effectors. The type 2 adenylyl cyclase can be stimulated by G protein βγ-dimers when it is preactivated by $G\alpha_s$·GTP (Federman et al., 1992) or by protein kinase C-mediated phosphorylation (Tsu and Wong, 1996). It is conceivable that similar mechanisms exist for the regulation of PLCβ. This might explain why βγ-complexes can participate in the stimulation of PLCβ when $G\alpha_{16}$ was coexpressed with DOR, but cannot do so in the absence of $G\alpha_{16}$. By drawing an analogy to the type 2 adenylyl cyclase system, we tested whether preactivation of PLCβ allows G_i-linked receptors to subsequently stimulate PLCβ. To induce preactivation of PLCβ, COS-7 cells were cotransfected with cDNAs encoding DOR and $G\alpha_{16}$QL, a constitutively activated mutant of $G\alpha_{16}$ (Heasley et al., 1996). Because $G\alpha_{16}$QL is "locked" in the GTP-bound state, it is relatively unresponsive to activation by DOR compared with $G\alpha_{16}$ wild-type. In the absence of any opioid agonist, $G\alpha_{16}$QL-expressing cells exhibited higher basal PLCβ activity, which is indicative of the constitutive activity of $G\alpha_{16}$QL (Fig. 3). Application of 100 nM DPDPE to the transfected cells further enhanced the IP formation by ~90%. The DPDPE-induced enhancement was completely PTX-sensitive (Fig. 3), indicating the involvement of G_i proteins instead of G_{16}. These results imply that when PLCβ is activated by $G\alpha_{16}$QL, it may then become responsive to stimulation by G_i-linked receptors through a G_i-mediated mechanism. This mechanism might involve the βγ-subunits because coexpression of $\beta_1\gamma_2$ with $G\alpha_{16}$QL significantly increased the basal PLCβ activity by 25.7 ± 2.9% ($n = 4$; $P < .05$ by paired t test) compared with that obtained with the expression of $G\alpha_{16}$QL alone. No enhancement of $G\alpha_{16}$QL activity was observed by coexpressing the nonfunctional $\beta_3\gamma_2$-complex.

Because DOR can use $G\alpha_{16}$ to stimulate PLCβ (Lee et al., 1998), it is conceivable that the DPDPE-induced stimulation was due to improved maintenance of $G\alpha_{16}$QL in the active state. To eliminate this possibility, we repeated the experiment with a constitutively activated mutant of $G\alpha_q$ ($G\alpha_q$RC; Conklin et al., 1992), which should not interact with DOR. As shown in Fig. 4, coexpression of DOR and wild-type $G\alpha_q$ in COS-7 cells did not permit DPDPE to stimulate IP accumulation. In contrast, coexpression of $G\alpha_q$RC raised the basal accumulation of IP by 8-fold, and activation of DOR further increased the IP formation (Fig. 4). Again, the DPDPE-induced stimulation of PLCβ in the presence of $G\alpha_q$RC was PTX-sensitive (Fig. 3). In contrast, replacement of $G\alpha_q$RC by the constitutively active mutant of $G\alpha_s$ ($G\alpha_s$RC) did not allow DPDPE to stimulate the PLCβ activity (Fig. 4). Additional experiments using the constitutively active mutant of $G\alpha_{14}$ ($G\alpha_{14}$RC) yielded similar results, except that the DPDPE-induced response was not completely abolished by PTX (Fig. 3). The reason for the incomplete blockade of the DPDPE-induced response by PTX is unclear. Nevertheless, these data suggest that preactivation of PLCβ by constitutively active mutants of $G\alpha_q$, $G\alpha_{14}$, or $G\alpha_{16}$ permits DOR to stimulate PLCβ in a PTX-sensitive manner.

Next, we asked if such permissive stimulation of PLCβ by DOR is dependent on the extent of preactivation. COS-7 cells were transiently cotransfected with cDNAs encoding DOR (0.25 μg/ml) and varying amounts of $G\alpha_{14}$RC cDNA up to 5 μg/ml. Transfected cells were then assayed for IP formation in the absence or presence of 100 nM DPDPE. No direct correlation was observed between the magnitude of the DPDPE-induced stimulations and the extent of preactivation of PLCβ by the mutationally activated Gα-subunits; the magnitude of the DPDPE-induced enhancement did not correspond with the level of expression of $G\alpha_{14}$RC (Fig. 5). Replacement of $G\alpha_{14}$RC by $G\alpha_q$RC or $G\alpha_{16}$QL produced similar results (data not shown). To examine if these DPDPE-induced enhancements exhibit agonist dose-dependence, we cotransfected COS-7 cells with cDNAs encoding DOR and one of the three constitutively active mutants. The cDNA concentration for the constitutively active mutants was lowered to 0.1 μg/ml to enhance the signal-to-noise ratio. The transfected cells were stimulated with varying concentrations of

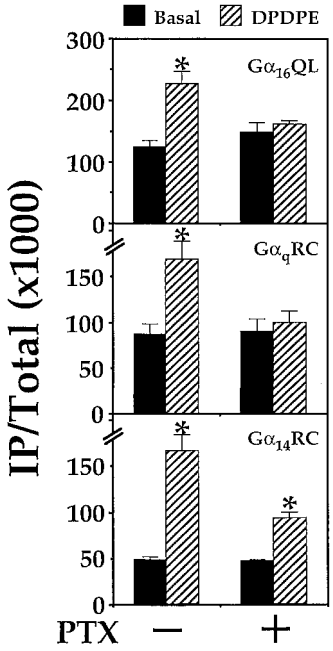

Fig. 3. Constitutively active Gα-mutants permit stimulation of PLCβ by DOR. COS-7 cells were cotransfected with 0.25 μg/ml DOR and one of the following cDNAs: $G\alpha_{16}$QL, $G\alpha_q$RC, or $G\alpha_{14}$RC (all at 0.15 μg/ml). After labeling with or without 100 ng/ml PTX, IP accumulation was assayed in the absence (basal) or presence of 100 nM DPDPE. *, DPDPE significantly increased IP production beyond basal levels; $n = 3$, Bonferroni t test, $P < .05$.

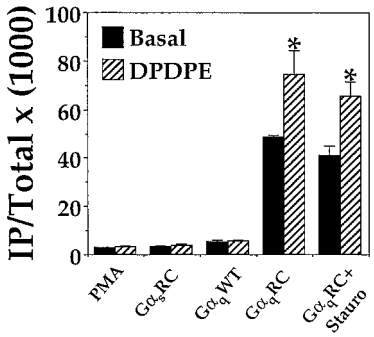

Fig. 4. DPDPE-induced PLCβ-activation is protein kinase C-independent. COS-7 cells were transfected with 0.25 μg/ml DOR alone or together with one of the following: $G\alpha_s$RC (0.25 μg/ml), $G\alpha_q$WT (0.25 μg/ml), or $G\alpha_q$RC (0.1 μg/ml). After labeling with [^{3}H]myo-inositol, cells transfected with DOR alone were treated with 100 nM PMA. Where indicated, DOR/$G\alpha_q$RC-transfected cells were pretreated with 500 nM staurosporin (stauro). After 15 min of pretreatment, cells were assayed for IP accumulation in the absence (basal) or presence of 100 nM DPDPE. *, DPDPE significantly enhanced IP production over basal values; $n = 3$, Bonferroni t test, $P < .05$.

DPDPE (ranging from 0.3 to 300 nM). DPDPE dose-dependently stimulated IP formation in all three cases (Fig. 6). In the presence $G\alpha_qRC$, the EC_{50} for the DPDPE-induced response was ~50 nM. The EC_{50} values for the DPDPE-induced response were ~10 to 20 nM for $G\alpha_{16}QL$ and $G\alpha_{14}RC$ transfected cells, and were slightly lower than that obtained with their wild-type counterparts (~40 nM; Lee et al., 1998). Such studies demonstrate that agonist-dependent activation of DOR in the presence of constitutively active G_q subfamily mutants can potentiate PLCβ-activity.

In the activation of type 2 adenylyl cyclase, phosphorylation by protein kinase C is one of the permissive conditions under which $\beta\gamma$-dimers can stimulate the production of cAMP (Tsu and Wong, 1996). Because the constitutive activity of $G\alpha_qRC$ (as well as $G\alpha_{16}QL$ and $G\alpha_{14}RC$) leads to the activation of PLCβ and subsequently protein kinase C, we asked if this permits $\beta\gamma$-dimers to further stimulate PLCβ in a manner similar to that observed for type 2 adenylyl cyclase. We began by substituting $G\alpha_qRC$ with phorbol-12-myristate-13-acetate (PMA), a direct activator of protein kinase C. In COS-7 cells expressing DOR alone, prior treatment with 100 nM PMA for 15 min did not allow DPDPE to stimulate PLCβ (Fig. 4). Moreover, 500 nM staurosporin (a protein kinase C inhibitor) did not prevent DPDPE from stimulating PLCβ in cells coexpressing DOR and $G\alpha_qRC$ (Fig. 4). Hence, the DOR-induced, $G\alpha_qRC$-dependent stimulation of PLCβ did not seem to require the activation of protein kinase C.

To extend our findings beyond DOR, we assayed for G_i-mediated stimulation of PLCβ by two other receptors in the presence of $G\alpha_qRC$. The fMLP and ORL_1 receptors are incapable of coupling to $G\alpha_q$ (Tsu et al., 1995a; Yung et al., 1999). Each receptor was coexpressed with either $G\alpha_q$ or $G\alpha_qRC$ in COS-7 cells and then assayed for agonist-induced stimulation of IP formation. In cells coexpressing the fMLP receptor and $G\alpha_qRC$, 200 nM fMLP stimulated the production of IP in a PTX-sensitive manner (Fig. 7). Replacement of $G\alpha_qRC$ with the wild-type $G\alpha_q$ effectively abolished the fMLP-induced stimulation of IP accumulation. The presence of $G\alpha_qRC$ also was required for the ORL_1 receptor-mediated stimulation of PLCβ (Fig. 7). Interestingly, the stimulatory response induced by 100 nM nociceptin was insensitive to PTX treatment (Fig. 7). This result indicated that the ORL_1 receptor might use PTX-insensitive G proteins to release $\beta\gamma$-dimers and mediate the $G\alpha_qRC$-dependent stimulation of PLCβ. Indeed, there is indirect evidence to implicate the association of the ORL_1 receptor to the PTX-insensitive $G\alpha_{12}$ (Yung et al., 1999), which is present in COS-7 cells. It also should be noted that the apparent insensitivity of the nociceptin response to PTX treatment was partly due to the high basal PLCβ-activity induced by $G\alpha_qRC$. Irrespective of their PTX sensitivity, the $G\alpha_qRC$-dependent stimulation of PLCβ was not limited to DOR, and could be extended to include other G_i-linked receptors.

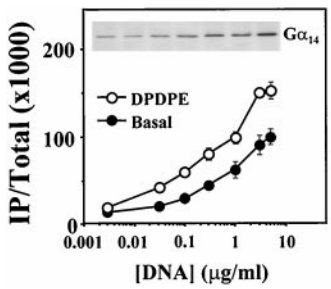

Fig. 5. cDNA dose-dependence of $G\alpha_{14}RC$-mediated permissive activation of PLCβ by DOR. COS-7 cells were cotransfected with DOR (0.25 μg/ml), and varying concentrations of $G\alpha_{14}RC$ cDNAs (3 ng/ml to 5 μg/ml). Cells were then labeled and assayed with or without 100 nM DPDPE. Inset shows the expression of $G\alpha_{14}RC$ in cells transfected with the seven different doses of cDNA was determined by Western blot analysis with a $G\alpha_{14}$-specific antiserum 3A-195.

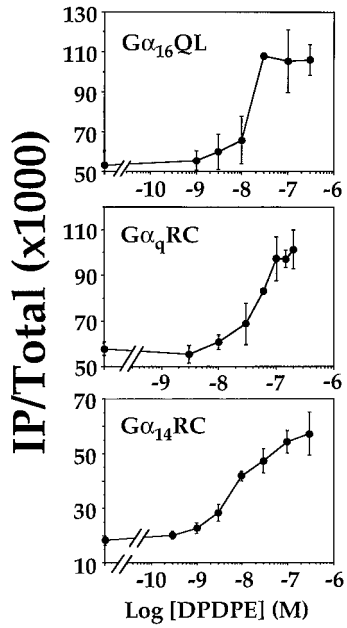

Fig. 6. DPDPE dose-dependently increases IP formation in cells coexpressing DOR and mutants of $G\alpha_q$, $G\alpha_{16}$, or $G\alpha_{14}$. COS-7 cells were cotransfected with cDNAs encoding DOR (0.25 μg/ml) and $G\alpha_qRC$, $G\alpha_{16}QL$ (both at 0.1 μg/ml), or $G\alpha_{14}RC$ (0.25 μg/ml). The cells were labeled and stimulated with varying concentrations of DPDPE (0–200 nM for $G\alpha_qRC$ and 0–300 nM for $G\alpha_{16}QL$ or $G\alpha_{14}RC$).

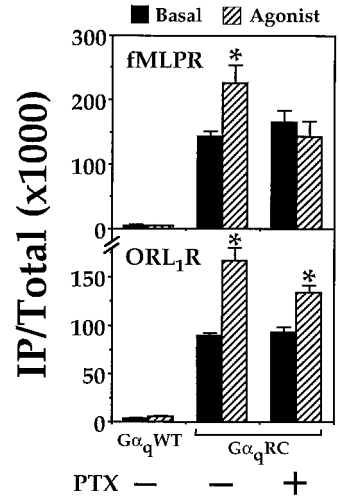

Fig. 7. Permissive activation of PLCβ by other G_i-coupled receptors in the presence of $G\alpha_qRC$. COS-7 cells were cotransfected either with $G\alpha_qWT$ (0.25 μg/ml) or $G\alpha_qRC$ (0.1 μg/ml) and either the fMLP or ORL_1 receptor cDNAs (both at 0.25 μg/ml). After transfection, the cells were labeled in the absence or presence of 100 ng/ml PTX as indicated. IP accumulation was assayed in the absence (basal) or presence of agonist (200 nM fMLP or 100 nM nociceptin). *, agonist-induced responses were significantly higher than the corresponding basal values; $n = 3$, Bonferroni t test, $P < .05$.

Synergism between G_i- and G_q-Linked Receptors on PLCβ-Activity. Given that G_i-linked receptors can stimulate PLCβ-activity in the presence of $G\alpha_q RC$, they should be able to enhance signals derived from the activation of G_q-linked receptors. We tested this hypothesis by substituting $G\alpha_q RC$ with either an endogenously or recombinantly expressed G_q-linked receptor. COS-7 cells were found to endogenously express a purinergic P2Y receptor. Over a concentration range from 0.1 to 300 μM, ATP dose-dependently stimulated the formation of IP (data not shown but they were similar to those presented in Fig. 8). Preliminary characterization with selective antagonists indicated that this purinergic receptor belongs to the $P2Y_2$ class (North and Barnard, 1997); suramin hexasodium blocked the ATP-induced stimulation of IP formation, whereas pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium was ineffective (data not shown). The $P2Y_2$ receptor is known to stimulate PLCβ via $G_{q/11}$ proteins and is expressed in the kidney (Lustig et al., 1993b), which is the tissue origin of COS-7 cells. In COS-7 cells transiently expressing DOR, DPDPE alone did not stimulate IP formation (Lee et al., 1998), whereas ATP dose-dependently elevated the IP levels by ~4-fold with an EC_{50} of ~6 μM (Fig. 8). When both δ-opioid and $P2Y_2$ receptors were simultaneously activated, the resultant IP accumulation was significantly greater than that obtained by activating $P2Y_2$ receptors alone (Fig. 8). Addition of 100 nM DPDPE to the ATP dose-response curve raised the maximal stimulation by ~65% with no change on the EC_{50} value (~5 μM; Fig. 8). The DPDPE-induced enhancement of the $P2Y_2$ receptor-mediated stimulation of PLCβ was completely inhibited by PTX (Fig. 9).

The observed synergism between the $P2Y_2$ and DOR suggest that DOR also may synergise with other G_q-linked receptors. Thus, we cotransfected COS-7 cells with cDNAs encoding DOR and the G_q-linked bombesin receptor. Bombesin alone stimulated IP formation in a dose-dependent manner with an EC_{50} of ~1 nM (Fig. 8). Similar to the endogenous $P2Y_2$ system, inclusion of 100 nM DPDPE elevated the maximal bombesin response by ~50% but it did not alter the EC_{50} value (~1 nM; Fig. 8). Replacement of the bombesin receptor with another G_q-linked receptor, the m1 muscarinic receptor, produced similar results. DPDPE enhanced the carbachol-induced stimulation of IP formation (Fig. 8). In both cases, the DPDPE-induced enhancement was totally abolished by PTX (Fig. 9). Collectively, these results show that simultaneous activation of G_q- and G_i-linked receptors can produce a more efficient stimulatory control on PLCβ.

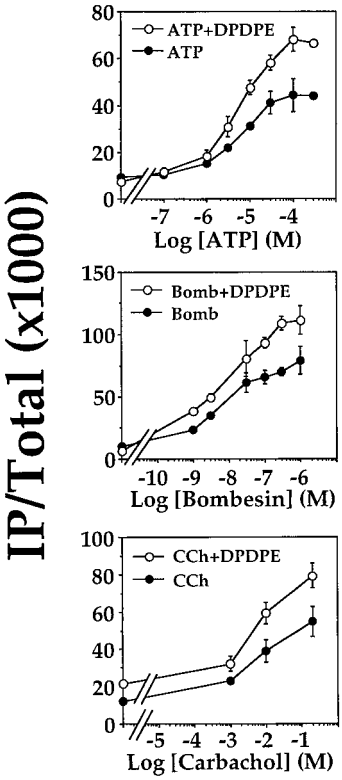

Fig. 8. Synergistic activation of PLCβ by G_q- and G_i-coupled receptors. COS-7 cells were transfected with DOR alone (top) or together with either the bombesin receptor or muscarinic m1 receptor (all cDNAs at 0.25 μg/ml). After transfection, the cells were labeled overnight and assayed for IP accumulation with varying concentrations of ATP (0–300 μM; the agonist for endogenous $P2Y_2$ receptors), bombesin (0–1 μM), or carbachol (0–200 μM; the m1 receptor agonist), with or without 100 nM DPDPE. Agonist stimulation of PLC with or without DPDPE was in all cases dose-dependent. DPDPE increased the maximal response of G_q-coupled receptor agonists alone.

Discussion

The complex network of signal transduction pathways regulated by GPCRs must possess critical loci for signal integration and processing. Synergistic cross talk interactions between G_i/G_s- and G_q-coupled receptors may provide a mechanism for the fine-tuning of signals generated from GPCRs. For example, activation of the G_i-linked adenosine A_1 receptor augments the stimulation of PLCβ evoked by G_q-linked receptors such as α_1-adrenergic, bradykinin, his-

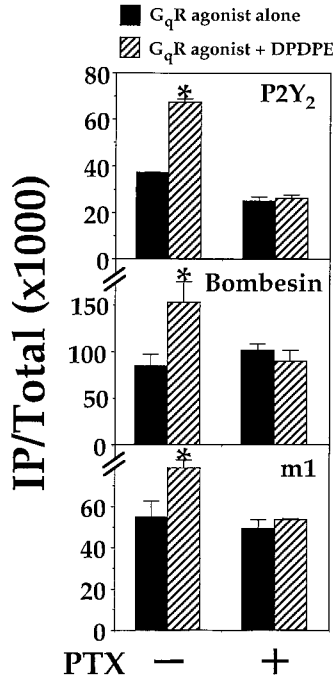

Fig. 9. The synergistic effect produced by DPDPE is abolished by PTX. COS-7 cells were transfected as in Fig. 8 but treated with or without 100 ng/ml PTX as indicated. IP production was assayed with 100 μM ATP, 100 nM bombesin, or 200 μM carbachol with or without 100 nM DPDPE. *, DPDPE significantly enhanced IP production; $n = 3$, Bonferroni t test, $P < .05$. The DPDPE induced increase in IP accumulation was in all cases abolished by PTX.

tamine H_1, and muscarinic receptors (for review, see Selbie and Hill, 1998). Often, stimulation of G_i-coupled receptors alone has no effect, but augments G_q-mediated responses when both are stimulated concurrently. The present study provides a mechanistic basis for synergistic cross talk between G_q- and G_i-coupled receptors because preactivation of PLCβ by the α-subunit of G_q subfamily members apparently allows $\beta\gamma$-dimers to further stimulate PLCβ.

Several observations support our notion that preactivation of PLCβ permits subsequent stimulation by G_i-coupled receptors. First, DOR could not stimulate PLCβ in COS-7 cells unless activated Gα-subunits of the G_q subfamily are available. Active Gα-subunits can be provided in the form of constitutively active mutants (Gα_qRC, Gα_{16}QL, and Gα_{14}RC) or generated through receptor coupling to the promiscuous G_{16}. Second, the constitutively active Gα_s-mutant did not permit DPDPE to stimulate PLCβ. Third, in the presence of a mutationally activated α-subunit of the G_q subfamily, DPDPE-induced enhancement of IP production occurs in a dose-dependent and PTX-sensitive manner. Fourth, concurrent activation of G_q- and G_i-linked receptors resulted in significantly higher PLCβ activities compared with stimulating a G_q-linked receptor alone. And fifth, the G_{16}-dependent stimulation of PLCβ by DOR was partially sensitive to PTX, suggesting dual mechanisms of activation of PLCβ. Collectively, these results indicate that PLCβ can integrate coincident signals in much the same way as the type 2 adenylyl cyclase, where prior activation by one type of signal allows subsequent detection of other signals.

The mechanism by which G_i-linked receptors synergise with Gα_q-mediated stimulation of PLCβ appears to involve the $\beta\gamma$-dimer. It is well established that the $\beta\gamma$-dimer can directly activate PLCβ1- to β3-isozymes and the sensitivity of PLCβ-isozymes to $\beta\gamma$-subunits decreases in the order PLCβ3 > β2 > β1 (for review, see Rhee and Bae, 1997). In the present study, there are strong indications for the involvement of $\beta\gamma$-subunits in mediating the synergistic effects of G_i- and G_q-coupled receptors on PLCβ. The synergistic effect can be potently inhibited by Gα_{t1}, a known scavenger of $\beta\gamma$-subunits. Moreover, the EC_{50} for DPDPE to stimulate PLCβ in the presence of activated Gα_q-subunits (10–50 nM; Fig. 6) is much higher than that required for its inhibitory effect on adenylyl cyclase (~1 nM; Tsu et al., 1995b). This is in agreement with the concept that the EC_{50} for a $\beta\gamma$-mediated response is considerably higher than responses mediated through the corresponding α-subunits (Iñiguez-Lluhi et al., 1993). Likewise, adenosine A_1 receptor-mediated potentiation of PLCβ activity in cultured astrocytes requires higher concentrations of agonist than for adenylyl cyclase inhibition (Biber et al., 1997).

It is not clear by which mechanism preactivation of PLCβ allows subsequent stimulation by $\beta\gamma$-subunits. Activation of PLCβ by Gα_q requires the C-terminal extension unique to the β-isozymes (Park et al., 1993; Wu et al., 1993), whereas $\beta\gamma$ interacts with the pleckstrin homology and EF-hand domains (Kuang et al., 1996). Reconstitution experiments have demonstrated that the effects of Gα_q and $\beta\gamma$ on PLCβ3 are additive (Smrcka and Sternweis, 1993). The present study provides evidence that, in intact cells, regulation of PLCβ by activated Gα_q may modulate its responsiveness to $\beta\gamma$-dimers. Structural information on the PLCβ-isozymes in the future will hopefully resolve how the binding of Gα_q might modulate the $\beta\gamma$-binding site.

Unlike the type 2 adenylyl cyclase, phosphorylation of PLCβ by protein kinase C does not permit subsequent stimulation by $\beta\gamma$-dimers. Although avian PLCβ is phosphorylated by protein kinase C in vivo, it is accompanied by a concomitant loss of enzyme activity (Filtz et al., 1999). Because the role of Gα_qRC in permitting DOR to stimulate PLCβ could not be substituted by phorbol ester treatment, and that the response was not suppressed by staurosporin, the G_i-mediated enhancement did not seem to require the activation of protein kinase C. However, these studies do not exclude the possibility that protein kinase C can regulate

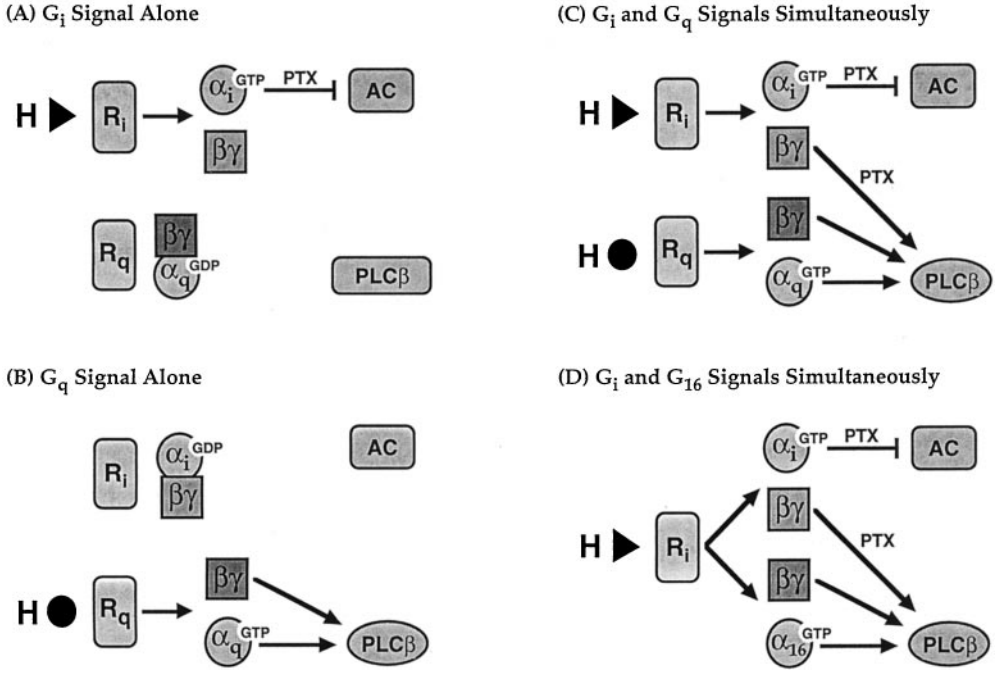

Fig. 10. Mechanistic models in which G_i-coupled receptors can activate PLCβ in the absence or presence of costimulation by G_q-coupled receptors. Four models (A-D) for the activation of PLCβ by G_i-released $\beta\gamma$-subunits are depicted. For simplicity, the adenylyl cyclase (AC) isoforms are assumed to be nonresponsive to $\beta\gamma$-dimers. H, hormone; R_i, G_i-coupled receptor; R_q, G_q-coupled receptor. Arrows indicate activation of signaling pathways. Inactive PLCβ is shown as a rectangle, whereas activated isoforms are illustrated as ovals.

long-term potentiation of PLCβ-activity (Schmidt et al., 1998) or suppress receptor-induced stimulation of PLCβ mediated via $G\alpha_{16}$ (Aragay and Quick, 1999).

It is well documented that in many cells and tissues, G_i- and G_s-coupled receptors rarely stimulate PLCβ on their own. COS-7 cells endogenously express the PLCβ1 and PLCβ3, but not PLCβ2 (Katz et al., 1992). Both β1 and β3 isoforms can be efficiently stimulated by $G\alpha_q$, but only PLCβ3 can exhibit augmentation by βγ-dimers (Smrcka and Sternweis, 1993). With a 10-fold greater potency, only $G\alpha_q$-mediated signals can efficiently stimulate PLCβ in COS-7 cells. If preactivation facilitates the βγ-mediated stimulation of PLCβ, then those G_i-coupled receptors that possess a weak ability to activate G_q will be able to induce a PLCβ-response. An example of such an occurrence is the α_2-adrenergic receptor (Conklin et al., 1992). Efficient stimulation of PLCβ2 by G_i-linked receptors in some cell types (e.g., HL-60; Camps et al., 1992; Katz et al., 1992) suggests that this isoform probably does not require preactivation for βγ-mediated stimulation. Supportive evidence from fluorescence spectroscopy indicates that βγ-dimers bind to PLCβ2 more tightly than to β1 or β3 (Runnels and Scarlata, 1999). Moreover, it has been shown that recombinant $G\alpha_{16}$ and $G\alpha_q$ do not change the sensitivity of PLCβ2 to stimulation by βγ-dimers (Kozasa et al., 1993). Whether preactivation-dependent, βγ-mediated stimulation of PLCβ is generally applicable to β1–3 isoforms, or if these isozymes are indeed differentially regulated, would require further studies.

The need of preactivation for βγ-dimers to efficiently stimulate PLCβ in intact cells has major mechanistic implications on signal processing via the PLCβ-pathway. In the absence of stimulation by $G\alpha_q$, PLCβ (perhaps except the β2 subtype) is relatively nonresponsive to free βγ-dimers, hence activation of G_i-coupled receptors will only lead to the regulation of adenylyl cyclase (Fig. 10A). This might explain why many G_i-coupled receptors require the coexpression of PLCβ2 in COS-7 cells to manifest a βγ-mediated stimulation of IP formation (Lee et al., 1993). Activation of a G_q-coupled receptor will generate two signaling components, $G\alpha_q$- and the βγ-dimer, that exhibit differential abilities to stimulate PLCβ (Fig. 10B). Costimulation of G_i- and G_q-linked receptors will produce a stronger stimulation of PLCβ because the βγ-subunits released from G_i activation can now augment the G_q-derived signal (Fig. 10C). The G_i-linked adenosine A_1 receptor can certainly augment IP signals generated from a variety of G_q-linked receptors (Selbie and Hill, 1998). The present study shows that DOR can augment the PLCβ-activities evoked by purinoceptor, muscarinic, or bombesin receptors. In the central nervous system, cholecystokinin has been reported to enhance the analgesic potentials of opioid peptides (Noble et al., 1993). Because the cholecystokinin receptors are typically coupled to G_q, and opioid receptors are associated with G_i proteins, it is conceivable that PLCβ may act as the point of signal convergence in neurons where both receptors are colocalized. Last, a single receptor that can activate both G_i and G_{16} should be able to stimulate the PLCβ-activity efficiently (Fig. 10D). Because part of the signal is derived from G_i, the overall response should be partially sensitive to PTX treatment. Examples of such observations can be readily found. $G\alpha_{16}$-dependent signaling by the $P2Y_1$ purinoceptor (Baltensperger and Porzig, 1997) and leukotriene B_4 receptor (Gaudreau et al., 1998) are indeed partially sensitive to PTX. These results are in good agreement with our findings on the PTX sensitivity of the G_{16}-mediated stimulation of PLCβ by DOR. Although G_{16} is not expressed in the central nervous system, it colocalizes with neuropeptide receptors, such as the opioid receptors, in a number of hematopoietic cells. The mechanism depicted in Fig. 10D may in fact be applicable to neuropeptides involved in the modulation of immune and endocrine responses.

In conclusion, this study provides evidence that augmentation of G_q-stimulated PLCβ-activity by G_i-linked receptors requires preactivation. The proposed mechanism resembles the one used by type 2 adenylyl cyclase. Signal integration by cells or neurons is a complex and delicate process that often requires fine-tuning to discern an array of incoming signals. Temporal summation of various signals allows a cell to reinforce critical inputs and perhaps to establish itself as part of a distinct neural circuit. We envisage that many G_i- and G_s-coupled receptors when costimulated with G_q-coupled receptors can produce synergistic actions on PLCβ in neurons and other target cells.

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