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$G\alpha_{14}$ links a variety of G_i- and G_s-coupled receptors to the stimulation of phospholipase C

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1 The bovine $G\alpha_{14}$ is a member of the G_q subfamily of G proteins that can regulate phospholipase Cβ isoforms but the extent to which $G\alpha_{14}$ recognizes different receptor classes is not known.

2 $G\alpha_{14}$ was cotransfected with a variety of receptors in COS-7 cells, and agonist-induced stimulation of phospholipase C was then measured.

3 Activation of the type 2 but not type 1 somatostatin receptor in cells coexpressing $G\alpha_{14}$ stimulated the accumulation of inositol phosphates; functional expression of both subtypes of somatostatin receptors was determined by the ability of somatostatin to inhibit cyclic AMP accumulation.

4 Among the three opioid receptors (μ, δ, and κ), only the δ receptor was capable of stimulating IP formation when coexpressed with $G\alpha_{14}$ in COS-7 cells.

5 A panel of G_i- and G_s-linked receptors was screened for their ability to stimulate IP accumulation *via* $G\alpha_{14}$. The adenosine A_1, complement C5a, dopamine D_1, D_2 and D_5, formyl peptide, luteinizing hormone, secretin, and the three subtypes of melatonin (mt1, MT2, and *Xenopus*) receptors were all incapable of activating $G\alpha_{14}$, while the α_2- and β_2-adrenoceptors were able to do so.

6 $G\alpha_{14}$-mediated stimulation of phospholipase Cβ was agonist dose-dependent. These data demonstrate that although $G\alpha_{14}$ can interact with different classes of receptors, it is much less promiscuous than $G\alpha_{15}$ or $G\alpha_{16}$.

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Abbreviations: 5-HT, 5-hydroxytryptamine; β_2AR, β_2-adrenergic receptor; COS-7, simian kidney fibroblasts; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; DMEM, Dulbecco's modified Eagle's medium; fMLP, *N*-formylmethionylleucylphenylalanine; GPCRs, G protein-coupled receptors; G protein, guanine nucleotide-binding regulatory protein; hCG, human choriogonadotropin; IP, inositol phosphates; LHR, luteinizing hormone receptor; ORL_1, opioid receptor-like receptor; PAF, platelet-activating factor; PIA, (+)-N^6-(2-phenylisopropyl)-adenosine; PLCβ, phospholipase Cβ isoforms; PTX, pertussis toxin; SSTR, somatostatin receptor; U50,488H, *trans*-(±)-3,4-dichloro-N-methyl-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulphonate; UK14304, 5-bromo-N-(4,5-dihydro-1H-imidazol-2yl)-6-quinoxalinamine

Introduction

Cellular activities are often regulated and coordinated by extracellular signals such as hormones, neurotransmitters and growth factors. The G protein-coupled receptors (GPCRs) constitute the single largest family of signal detectors at the cell surface. Activation of GPCRs by specific ligands triggers signal propagation *via* the G proteins, which subsequently regulate the activities of downstream effector molecules (Bourne *et al*., 1990). The fidelity of GPCR-mediated signal transduction is maintained at several levels. Firstly, the ligand-receptor interaction is highly selective where discrimination of stereoisomers is commonly observed. Secondly, each GPCR can only interact with a small subset of G proteins, which in turn regulate a limited number of effectors. The G proteins are classified into four subfamilies termed G_s, G_i, G_q and G_{12}, according to their sequence homologies (Simon *et al*., 1991). The G_q subfamily is especially important in the regulation of cell proliferation since a large number of mitogens are known to act on G_q-coupled receptors.

Molecular cloning of cDNAs has revealed that there are four types of α-subunits belonging to the G_q subfamily (Simon *et al*., 1991). The cDNAs of $G\alpha_q$ and $G\alpha_{11}$ were isolated from the mouse brain (Strathmann & Simon, 1990), while those of $G\alpha_{14}$ and $G\alpha_{15}$ were cloned from mouse lung and spleen libraries, respectively (Wilkie *et al*., 1991). The bovine $G\alpha_{L1}$ and human $G\alpha_{16}$, which are the homologues of $G\alpha_{14}$ and $G\alpha_{15}$, were subsequently isolated (Nakamura *et al*., 1991; Amatruda *et al*., 1991). The primary structures of all the G_q family α-subunits share high percentages of identity with each other (Simon *et al*., 1991) and they also share common functional properties. They can regulate the activity of phospholipase Cβ isoforms (PLCβ) through selective

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activation by GPCRs. This leads to an increase in the intracellular level of inositol phosphates (IP) in a pertussis-toxin insensitive manner (Lee *et al*., 1992). Most GPCRs exhibit some specificity with regard to recognizing Gα subunits within the G_q subfamily. For instance, all three subtypes of α_1-adrenergic receptors (1A, 1B, and 1C) can be linked to PLCβ *via* $G_{q/11}$, but only the α_{1B}-adrenergic receptor can interact with G_{16} (Wu *et al*., 1992). Conversely, receptors for interleukin-8 and complement C5a interact productively with G_{16} but cannot activate $G_{q/11}$ (Wu *et al*., 1993; Buhl *et al*., 1993). This suggests that there are differences among the members of the G_q family in terms of receptor coupling.

Compared to the other members of the G_q subfamily, $G\alpha_{14}$ is the least studied subunit. In reconstitution experiments, $G\alpha_{14}$ behaves like other G_q subfamily members to stimulate PLCβ (Nakamura *et al*., 1995). Unlike the ubiquitously expressed $G\alpha_q$ and $G\alpha_{11}$, the distribution of $G\alpha_{14}$ is more restricted (Nakamura *et al*., 1991; Wilkie *et al*., 1991). Northern analysis revealed that $G\alpha_{14}$ is mainly expressed in tissues such as pancreatic islets (Zigman *et al*., 1994), taste tissue (McLaughlin *et al*., 1994), spleen, lung, kidney, testis, bone marrow stromal cells and certain myeloid and progenitor B cells (Nakamura *et al*., 1991; Wilkie *et al*., 1991). In this regard, the restricted distribution of $G\alpha_{14}$ resembles $G\alpha_{15}$ and $G\alpha_{16}$, which are primarily found in hematopoietic cells (Amatruda *et al*., 1991). The identity of the GPCRs that utilize G_{14} for signal transduction in native tissues and cells is presently unclear. $G\alpha_{14}$ has been shown to link the type 3 somatostatin (Komatsuzaki *et al*., 1997), opioid receptor-like (ORL_1; Yung *et al*., 1998), histamine H_2 (Bernhard *et al*., 1996), and parathyroid hormone-related protein (Maeda *et al*., 1996) receptors to stimulation of PLCβ in heterologous expression systems. Such observations are intriguing because these GPCRs are primarily coupled to G_2 or G_i proteins. Precedence for promiscuous coupling to receptors is seen with G_{15} and G_{16}. A large number of GPCRs can interact productively with either G_{15} or G_{16} to activate PLCβ (Offermanns & Simon, 1995). Given that $G\alpha_{14}$ has a demonstrated capacity to link G_s- and G_i-coupled receptors to stimulation of PLCβ, in this study we examined its extent of 'promiscuity'. We show that $G\alpha_{14}$ interacts selectively with a number of G_s- and G_i-coupled receptors. In contrast to $G\alpha_{15}$ and $G\alpha_{16}$ which couple non-selectively to a wide variety of receptors (Offermanns & Simon, 1995), $G\alpha_{14}$ is less promiscuous.

Methods

Materials

The cDNA encoding the α subunit of bovine G_{14} ($G\alpha_{L1}$) was generously provided by Dr T. Nukada (Tokyo Institute of Psychiatry, Japan). GPCR cDNAs were kindly donated by Drs Roger Sunhara (University of Texas, Southern Medical Center) for human dopamine D_5 receptor, Graeme Bell (University of Chicago) for mouse type 3 somatostatin receptor, Gang Pei for human ORL_1 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, PRC), Richard Ye (The Scripps Research Institute) for human platelet activating factor receptor, and Shigekazu Nagata (Osaka University Medical School, Japan) for rat secretin receptor. Other cDNAs were obtained as previously described (Lee *et al*., 1998, Mody *et al*. 2000). Simian kidney fibroblasts COS-7 (ATCC CRL-1651) were obtained from the American Type Culture Collection. [^{3}H]-Adenine and [^{3}H]-*myo*-inositol were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.) and DuPont NEN (Boston, MA, U.S.A.), respectively. Plasmid purification columns were obtained from QIAGEN GmbH (Hilden, Germany). Antisera against $G\alpha_{14}$ (3A-195) was purchased from Gramsch Laboratories (Schwabhausen, Germany). Cell culture reagents were supplied by Life Technologies Inc. (Rockville, MD, U.S.A.) and all other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Drugs

Carbachol, complement C5a, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAGO), dopamine, [D-Pen^{2,5}]enkephalin (DPDPE), *N*-formylmethionylleucylphenylalanine (fMLP), 5-hydroxytryptamine (5-HT), human choriogonadotropin (hCG), 2-iodomelatonin, isoproterenol, nociceptin, (+)-N^6-(2-phenylisopropyl)-adenosine (PIA), platelet activating factor (PAF), secretin, somatostatin, *trans*-(±)-3,4-dichloro-N-methyl-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulphonate (U-50,488H), and 5-bromo-N-(4,5-dihydro-1H-imidazol-2yl)-6-quinoxalinamine (UK14304) were purchased from Sigma (St. Louis, MO, U.S.A.) and Tocris Cookson Ltd. (Avonmouth, Bristol, U.K.). Pertussis toxin (PTX) was purchased from List Biological Laboratories (Campbell, CA, U.S.A.).

cDNA transfection and cell culture

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v v^{-1}) foetal bovine serum, 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2. COS-7 cells were transfected as reported previously (Tsu *et al*., 1995). Briefly, COS-7 cells were seeded on 12-well plates at approximately 1×10^5 cells per well. One day later, cells were transfected with medium containing the desired cDNAs (0.25 μg ml^{-1}) along with 250 μg ml^{-1} DEAE-dextran and 100 μM chloroquine for up to 4 h at 37°C. The cells were shocked with 10% (v v^{-1}) dimethylsulphoxide in phosphate buffered saline for 1 min and then returned to growth medium. The efficiency of transfection was routinely monitored by coexpressing the β-galactosidase as a reporter.

Assay for IP formation and cyclic AMP accumulation

Transfected COS-7 cells were labelled 1 day later with 0.75 ml of inositol-free DMEM containing [^{3}H]-*myo*-inositol (2.5 μCi ml^{-1}) and 5% foetal bovine serum. Where necessary, PTX (100 ng ml^{-1}) was added together with the radiolabel. After 24 h of labelling, the cells were rinsed with 1 ml of assay medium (20 mM HEPES-buffered DMEM with 20 mM LiCl). Drugs treatments were performed at 37°C for 1 h. [^{3}H]-Inositol phosphates (IP) were separated on columns containing Dowex resin (AG1 × 8, formate form; Bio-Rad) as described previously (Tsu *et al*., 1995). For cyclic AMP accumulation assays, transfected COS-7 cells were labelled

with 0.5 ml of DMEM containing 1 μCi of [^{3}H]-adenine and 1% foetal bovine serum. Labelled cells were rinsed once with 1 ml of HEPES-buffered DMEM and incubated at 37°C for 30 min with 1 ml of HEPES-buffered DMEM containing 1 mM 1-methyl-3-isobutylxanthine and the indicated drugs. Intracellular levels of [^{3}H]-cyclic AMP were determined by sequential chromatography as described previously (Wong, 1994).

Immunodetection of $G\alpha_{14}$

COS-7 cells were cotransfected with a receptor cDNA in the absence or presence of $G\alpha_{14}$. Transfected cells were harvested 48 h later and resuspended in lysis buffer (50 mM Tris-HCl containing (mM): phenylmethylsulfonyl fluoride 1, benzamidine-HCl 1, EGTA 1, $MgCl_2$ 5, and DTT 1; pH 7.4). The cells were 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 using the Bio-Rad protein assay kit (Cambridge, MA, USA). For each sample, 50 μg of membrane proteins were separated on a 12.5% sodium dodecyl sulphate-polyacrylamide gel and electrophorectically transferred to polyvinylidene fluoride membranes. Protein markers were localized by Ponceau S staining. Antigen-antibody complexes were visualized using the enhanced chemiluminescence kit from Amersham Pharmacia Biotech.

Data analysis

[^{3}H]-IP was estimated by determining the ratios of [^{3}H]-IP to [^{3}H]-inositol plus [^{3}H]-IP as previously described (Tsu *et al*., 1995). Intracellular [^{3}H]-cyclic AMP level was expressed in a similar fashion (Wong, 1994). Absolute values for IP or cyclic AMP accumulation varied between experiments, but variability within a given experiment was less than 10% in general. Data shown in each figure represent the mean $\pm$ s.d. of three or more independent experiments performed in triplicates. Bonferroni or paired *t*-test with 95% confidence was adopted to verify the significance between different treatment groups within the experiments.

Results

$G\alpha_{14}$ couples the δ-opioid and type 2 somatostatin receptors to PLCβ

To explore the promiscuity of $G\alpha_{14}$, we assessed its ability to mediate agonist-induced stimulation of PLCβ by non-G_q-coupled receptors which are available in our laboratory. Amongst the few GPCRs which are known to interact with $G\alpha_{14}$, the type 3 somatostatin receptor (SSTR3; Komatsuzaki *et al*., 1997) and the opioid receptor-like receptor (ORL_1; Yung *et al*., 1998) are typically linked to G_i proteins. Hence, we started by examining the ability of $G\alpha_{14}$ to interact with two other G_i-coupled somatostatin receptors. The type 1 and type 2 somatostatin receptors (SSTR1 and SSTR2) share substantial homology to SSTR3 and utilize the PTX-sensitive G_i proteins for signal transduction. COS-7 cells were transfected with cDNAs encoding the SSTR1 or SSTR2 in the absence or presence of $G\alpha_{14}$ cDNA. Application of 100 nM somatostatin did not affect the basal levels of IP accumulation in cells transfected with SSTR1 alone (Figure 1). In COS-7 cells coexpressing the SSTR1 and $G\alpha_{14}$, 100 nM somatostatin did not alter the basal PLC activity (Figure 1). Unlike the SSTR3, SSTR1 was unable to interact productively with $G\alpha_{14}$. Increasing the cDNA of $G\alpha_{14}$ (up to 1 μg ml^{-1}) used in the transfection did not allow the SSTR1 to stimulate PLCβ (data not shown). SSTR2 is capable of stimulating PLCβ *via* a PTX-sensitive mechanism (Akbar *et al*., 1994) and thus in COS-7 cells expressing this receptor, 100 nM somatostatin stimulated IP formation by $\sim$2 fold (Figure 1). Nevertheless, the same concentration of somatostatin gave a much more robust stimulation of PLCβ ($\sim$5 fold over basal) in COS-7 cells coexpressing the SSTR2 and $G\alpha_{14}$ (Figure 1). The inability of SSTR1 to interact with $G\alpha_{14}$ was not due to a lack of expression of SSTR1 because under identical experimental conditions, activation of SSTR1 resulted in inhibition of cyclic AMP accumulation (Figure 2). In COS-7 cells coexpressing the SSTR1 and dopamine D_1 receptor, 100 nM somatostatin potently inhibited the dopamine-stimulated cyclic AMP accumulation (Figure 2). The magnitude of the SSTR1-mediated inhibition was comparable to that obtained with the SSTR2 in a similar set of experiments (both $\sim$50%; Figure 2). Similar findings have been reported in human embryonic kidney 293 cells (Garcia & Myers, 1994). Coexpression of $G\alpha_{14}$ did not affect the ability of either receptor to inhibit the dopamine-stimulated cyclic AMP accumulation. These results indicated that $G\alpha_{14}$ can discriminate the different subtypes of somatostatin receptors and does not influence the receptors' ability to inhibit adenylyl cyclase.

The receptors for opioid peptides share substantial homologies with the somatostatin receptors, and thus are

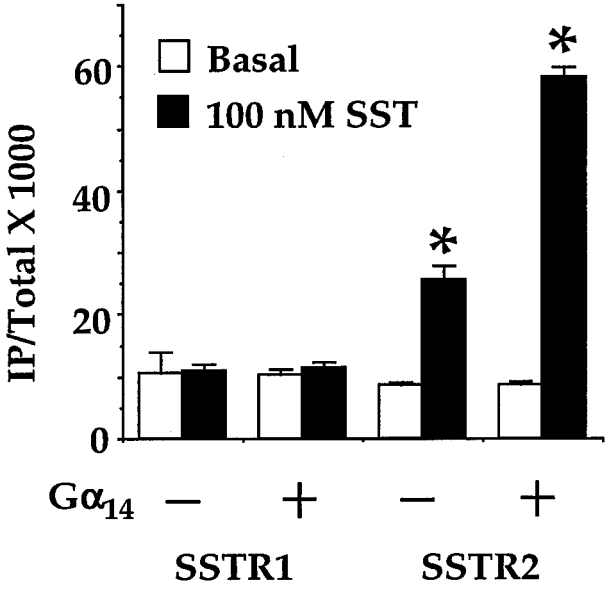

Figure 1 Differential coupling of $G\alpha_{14}$ to SSTR1 and SSTR2. COS-7 cells were transiently cotransfected with cDNAs encoding the SSTR1 or SSTR2 receptor with or without $G\alpha_{14}$. Transfected cells were labelled with [^{3}H]-inositol and subsequently assayed for the response to 100 nM of somatostatin (SST). *Somatostatin significantly stimulated IP production compared to the corresponding basal; Bonferroni *t*-test, $P<0.05$.

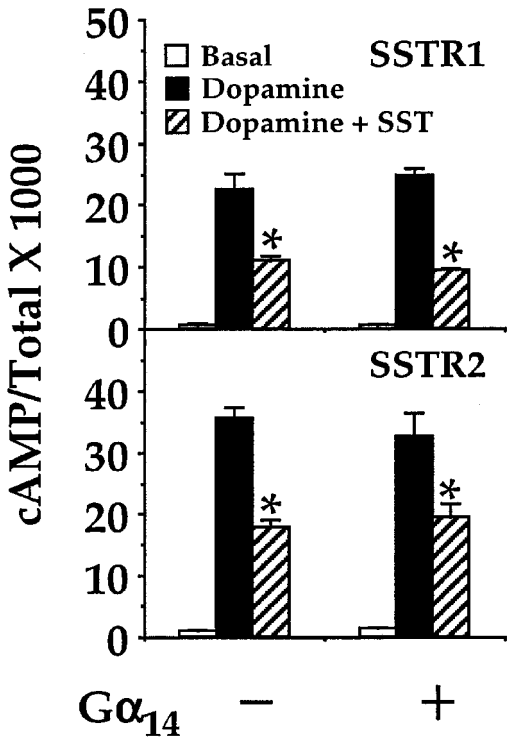

Figure 2 Coexpression of $G\alpha_{14}$ did not affect the ability of SSTR1 and SSTR2 to inhibit adenylyl cyclase. COS-7 cells were transiently cotransfected with cDNAs encoding the dopamine D_1 receptor, type 6 adenylyl cyclase, SSTR1 or SSTR2 with or without $G\alpha_{14}$. Transfected cells were labelled with [^{3}H]-adenine and assayed for cyclic AMP accumulation in the absence (basal) or presence of 1 μM dopamine with or without 100 nM of somatostatin as indicated. *Somatostatin significantly inhibited the dopamine-stimulated cyclic AMP accumulation; Bonferroni *t*-test, $P<0.05$.

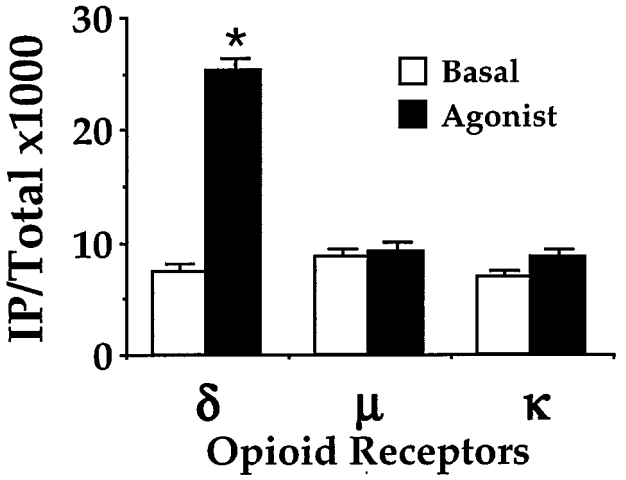

Figure 3 Differential coupling of $G\alpha_{14}$ to opioid receptors. COS-7 cells were transiently cotransfected with cDNAs, encoding the $G\alpha_{14}$ and one of the three opioid receptors: δ, μ, or κ subtype. Transfected cells were labelled with [^{3}H]-inositol and assayed for IP accumulation in the absence or presence of the appropriate opioid agonist (DPDPE for δ-receptor, DAGO for μ-receptor and U50,488H for κ-receptor) at 100 nM. *DPDPE significantly stimulated IP production compared to the corresponding basal; Bonferroni *t*-test, $P<0.05$.

potential candidates as upstream regulators of $G\alpha_{14}$. The three opioid receptor subtypes (μ, δ, and κ) are known to interact productively with both PTX-sensitive and -insensitive G proteins (Chan *et al*., 1995; Lai *et al*., 1995; Tsu *et al*., 1995). Differential coupling of the opioid receptors to $G\alpha_{16}$ has recently been demonstrated (Lee *et al*., 1998), though there is no report on their ability to utilize $G\alpha_{14}$ for signal transduction. We cotransfected COS-7 cells with equal amounts of cDNAs encoding one of the three opioid receptors and $G\alpha_{14}$. When transfected COS-7 cells were incubated with 100 nM of their respective opioid agonists (DPDPE for δ-receptor, U-50,488H for κ-receptor, and DAGO for μ-receptor), stimulation of PLCβ was only observed in cells cotransfected with the δ-opioid receptor and $G\alpha_{14}$ (Figure 3). IP formation was tripled in response to the δ-selective agonist, DPDPE. Activation of κ- and μ-opioid receptors did not result in elevated IP accumulation. Previous studies have shown that activation of all three opioid receptors produces equivalent degree of inhibition (50–60%) of cyclic AMP accumulation in transiently transfected cells (Chan *et al*., 1995; Lai *et al*., 1995; Tsu *et al*., 1995; Lee *et al*., 1998). The inability of κ- and μ-receptors to interact with $G\alpha_{14}$ cannot be rescued by increasing the cDNA concentrations of either the receptor or $G\alpha_{14}$ (data not shown). Radioligand binding assays on membranes prepared from the transfectants revealed that the differential abilities of $G\alpha_{14}$ to interact with opioid receptors were not due to variations in the expression levels of the opioid receptors. All three opioid receptors were expressed at comparable levels (B_{max} values $\sim$400–500 fmol mg^{-1} protein) similar to those reported previously (Lee *et al*., 1998). Like the somatostatin receptors, the opioid receptors possess different abilities to recognize $G\alpha_{14}$, and this differential coupling is more apparent than that observed with $G\alpha_{14}$ (Lee *et al*., 1998).

Next we determined the agonist dose-response relationship of the $G\alpha_{14}$-mediated stimulation of PLC by the SSTR2 and δ-receptors. COS-7 cells were cotransfected with cDNAs encoding $G\alpha_{14}$ and either the SSTR2 or δ-receptor. The transfected cells were assayed for IP accumulation in the presence of varying concentrations of the appropriate agonist. As shown in Figure 4, the SSTR2-mediated, $G\alpha_{14}$-dependent, stimulation of PLC occurred in a dose-dependent and saturable manner. Maximal stimulation occurred at around 100 nM of somatostatin with an EC_{50} of $\sim$10 nM. The EC_{50} values for somatostatin-induced stimulation of PLC (Akbar *et al*., 1994) and inhibition of adenylyl cyclase (Garcia & Myers, 1994) *via* the G_i-coupled SSTR2 were $\sim$50 and $\sim$0.2 nM, respectively. Likewise, in COS-7 cells coexpressing the δ-receptor and $G\alpha_{14}$, DPDPE dose-dependently stimulated the IP formation with an EC_{50} of $\sim$100 nM (Figure 4). The EC_{50} for DPDPE-induced, $G\alpha_{14}$-mediated stimulation of PLC was much higher than those required for G_i-mediated inhibition of adenylyl cyclase ($EC_{50}=1$ nM) or $\beta\gamma$-mediated activation of PLC ($EC_{50}=8$ nM) obtained in similar transfection studies (Tsu *et al*., 1995a). The high EC_{50} value resembles that of DPDPE-induced, $G\alpha_{16}$-mediated responses ($EC_{50}=40$ nM; Lee *et al*., 1998). These results indicate that the $G\alpha_{14}$-mediated stimulation of PLCβ is less efficient than the G_i-mediated inhibition of adenylyl cyclase.

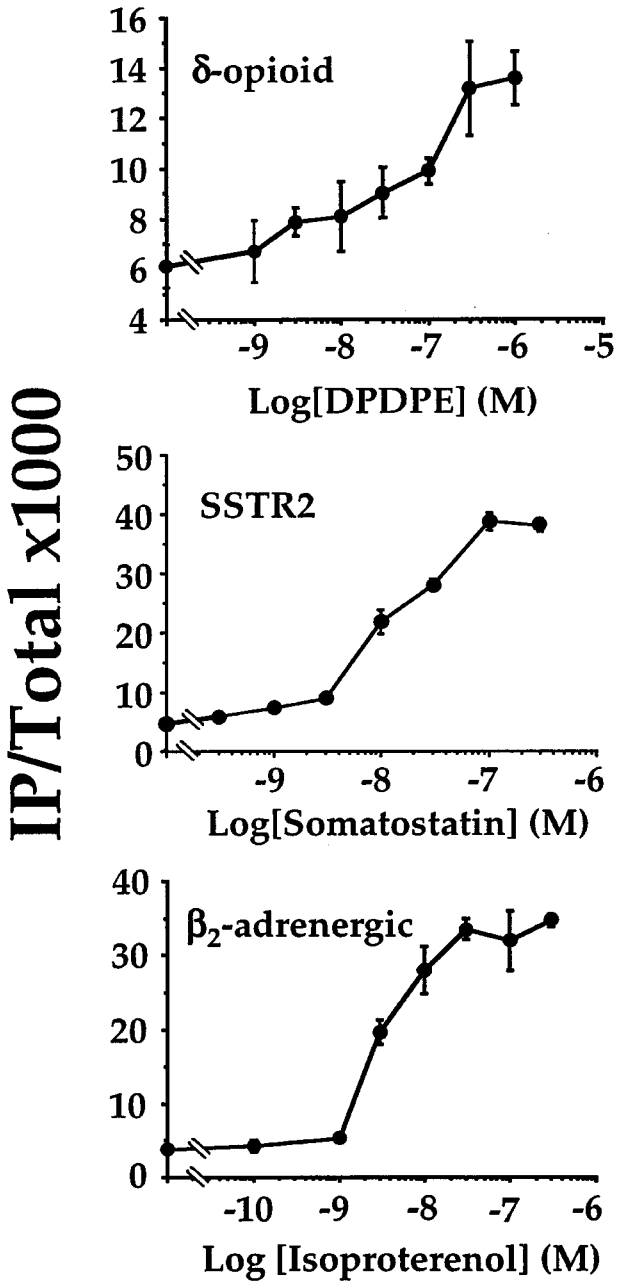

Figure 4 Agonist-dependent stimulation of PLCβ by Gα_{14}-coupled receptors. COS-7 cells were transfected with cDNAs encoding Gα_{14} and SSTR2, δ-opioid, or β_2-adrenergic receptors. Transfected cells were labelled and then assayed for IP formation in the presence of increasing concentrations of the indicated agonists.

Other G_i- and G_s-coupled receptors also stimulate PLC via Gα_{14}

Since Gα_{14} seemed to discriminate G_i-linked receptor subtypes within the same subfamily of GPCRs, we screened more G_i-coupled receptors for their capacity to activate Gα_{14}. Most of the GPCRs examined here were capable of stimulating Gα_{16}, and the Gα_{16}-mediated IP formation was included in Table 1 for comparison. COS-7 cells were cotransfected with Gα_{14} and a receptor chosen from a panel of G_i-coupled receptors that included both aminergic and peptidergic GPCRs. The adenosine A_1, α_2-adrenoceptor, dopamine D_2, complement C5a, formyl peptide (fMLP), and all three types of melatonin (mt1, MT2, and *Xenopus*) receptors were tested for their ability to activate Gα_{14} (Table 1). These receptors cannot stimulate PLCβ *via* endogenous $G_{q/11}$ (except when the α_2-adrenoceptor is overexpressed with Gα_q; Conklin *et al*., 1992). Receptors that can functionally interact with Gα_{14}, such as ORL_1 and SSTR3, were also included for comparison (Yung *et al*., 1998; Komatsuzaki *et al*., 1997). Transfected cells were assayed for IP formation in the absence or presence of saturating concentrations of the appropriate agonists. Coexpression of α_2-adrenoceptor and Gα_{14} allowed the selective agonist UK14304 to stimulate the IP accumulation by almost 4 fold (Table 1). In light of the notion that the α_2-adrenoceptor is relatively promiscuous (Conklin *et al*., 1992), its coupling to Gα_{14} is not surprising. In agreement with previous studies (Yung *et al*., 1998; Komatsuzaki *et al*., 1997), Gα_{14} mediated the ORL_1 and SSTR3-induced stimulation of PLCβ (Table 1). None of the remaining receptors were able to couple to Gα_{14} as revealed by the lack of agonist-induced PLCβ activation (Table 1). Under similar experimental paradigm, all of these receptors (except for the *Xenopus* melatonin receptor) are capable of stimulating PLCβ *via* Gα_{16}. Among the 15 G_i-coupled receptors tested in this study, only the SSTR2, SSTR3, α_2-adrenoceptor, δ-receptor, and ORL_1 receptors possessed the ability to interact productively with Gα_{14} (Table 1). G_{14} is thus much less promiscuous than G_{16}.

Both G_{15} and G_{16} possess the rare ability to recognize a wide range of GPCRs including those that are usually linked to G_s (Offermanns & Simon, 1995). Interestingly, G_{14} has also been reported to interact with the G_s-coupled histamine H_2 (Bernhard *et al*., 1996) and parathyroid hormone-related protein (Maeda *et al*., 1996) receptors. We therefore examined the ability of Gα_{14} to interact with a number of G_s-coupled receptors. Amongst the five G_s-coupled receptors that we have examined, only the β_2-adrenoceptor was capable of activating PLCβ *via* Gα_{14} (Table 1). The dopamine D_1 and D_5, luteinizing hormone and secretin receptors were all incapable of interacting with Gα_{14} (Table 1) despite the fact that these receptors stimulated cyclic AMP accumulation in the transfectants (data not shown) and can activate PLCβ when coexpressed with Gα_{16} (Table 1). Functional association of the β_2-adrenoceptor with Gα_{14} was somewhat surprising because a previous report failed to demonstrate coupling of the β_2-adrenoceptor to Gα_{14} (Wu *et al*., 1995a). To ensure that the β_2-adrenoceptor can indeed stimulate PLCβ via Gα_{14}, we cotransfected COS-7 cells with cDNAs encoding the β_2-adrenoceptor and Gα_{14} and then assessed the dose dependency of the isoproterenol-induced stimulation of PLCβ. As illustrated in Figure 4, isoproterenol stimulated IP formation in a dose-dependent and saturable manner with maximal stimulation occurring at 30 nM and an EC_{50} of $\sim$3 nM. Since the β_2-adrenoceptor can functionally associate with G_i proteins upon phosphorylation (Daaka *et al*., 1997), we asked if the observed stimulation of PLCβ activity was mediated partially or indirectly *via* G_i. To assess the involvement of G_i, we examined the ability of isoproterenol to stimulate PLCβ in COS-7 cells coexpressing the β_2-adrenoceptor and Gα_{14} with or without PTX treatment. PTX did not suppress the isoproterenol-induced stimulation of

Table 1 Coupling of $G\alpha_{14}$ and $G\alpha_{16}$ to various G_i- or G_s-coupled receptors

		IP formation					
			$G\alpha_{14}$			$G\alpha_{16}$	
Receptor	*Ligand*	*Basal*	*Agonist*	*% Response*	*Basal*	*Agonist*	*% Response*
G_i-coupled							
Adenosine A_1	10 μM PIA	8.1±1.1	7.0±1.1	86	10.4±0.8	61.4±3.5*	590*
α_2-Adrenergic	10 μM UK14304	8.4±0.4	40.0±0.9*	472*	9.6±1.0	108.2±7.6*	1127*
Complement C5a	100 nM C5a	6.0±0.3	7.7±1.2	128	8.8±0.2	69.1±2.8*	608*
Dopamine D_2	10 μM Dopamine	7.8±0.5	8.0±0.7	103	8.9±0.5	18.4±0.8*	207*
fMLP	200 nM fMLP	7.7±0.5	8.2±0.3	107	20.2±0.7	101.5±6.2*	502*
Melatonin mt1	1 μM 2-iodomelatonin	5.5±0.6	5.6±0.2	103	10.1±0.6	30.5±1.9*	302*
MT2	1 μM 2-iodomelatonin	5.2±0.5	4.9±0.5	94	9.4±0.7	17.7±1.3*	188*
Xenopus	1 μM 2-iodomelatonin	5.8±0.2	4.9±1.1	85	9.5±0.5	11.2±1.4	118
δ-Opioid	100 nM DPDPE	7.3±0.6	16.6±0.5*	226*	11.6±1.2	45.9±5.8*	396*
κ-Opioid	100 nM U-50,488H	6.9±0.4	8.7±0.7	126	8.0±0.2	22.9±1.8*	286*
μ-Opioid	100 nM DAGO	8.7±0.6	9.2±0.7	106	9.5±0.9	12.4±0.5*	131*
ORL_1	100 nM Nociceptin	5.3±0.7	27.1±1.4*	411*	12.7±1.6	87.1±5.4*	686*
SSTR1	100 nM Somatostatin	6.4±0.6	7.4±0.6	115	8.5±0.8	11.4±1.1*	134*
SSTR3	100 nM Somatostatin	8.9±0.7	41.6±4.0*	466*	9.0±0.7	25.9±2.0*	286*
G_s-coupled							
β_2-Adrenergic	10 μM Isoproterenol	8.2±0.3	28.8±1.0*	348*	13.2±0.6	59.8±4.7*	453*
Dopamine D_1	10 μM Dopamine	7.4±0.4	6.9±0.5	94	10.6±0.3	97.2±8.8*	917*
D_5	10 μM Dopamine	11.4±0.4	12.2±1.4	107	12.3±0.7	35.2±2.5*	287*
LHR	10 ng ml^{-1} hCG	6.7±0.2	6.4±0.8	96	9.2±0.4	27.7±1.9*	300*
Secretin	100 nM Secretin	15.1±2.8	17.1±1.1	113	11.4±2.8	25.1±1.0*	220*

*Agonist treatments significantly increased IP formation over basal levels; Bonferroni *t*-test, $P<0.05$.

PLCβ (Figure 5), indicating the lack of involvement of G_i proteins. Since the classical function of the β_2-adrenoceptor is to stimulate adenylyl cyclase, we also examined cyclic AMP accumulation. The ability of β_2-adrenoceptor to stimulate adenylyl cyclase was not affected by the coexpression of $G\alpha_{14}$ (Figure 5). Both the maximum response and the EC_{50} of isoproterenol from $G\alpha_{14}$-coexpressing cells were identical to those obtained with cells expressing the β_2-adrenoceptor alone. Although the β_2-adrenoceptor utilized $G\alpha_{14}$ to stimulate PLCβ, such interaction apparently did not affect the receptor's ability to activate $G\alpha_s$.

Several studies have indicated that $G\alpha_{14}$ can be activated by G_q-coupled receptors (Offermanns & Simon, 1995; Wu *et al*., 1995b). $G\alpha_{q/11}$-linked receptors such as the thrombin PAR1, thromboxane TP, vasopressin V_{1A}, 5-HT_{1C}, and α_{1B}-adrenoceptors exhibit enhanced ability to stimulate IP accumulation when coexpressed with $G\alpha_{14}$ in COS-7 cells. Similar enhancement of PLCβ activity was observed for the SSTR2 (Figure 1). We used the same strategy to test the ability of other $G\alpha_{q/11}$-linked receptors to activate $G\alpha_{14}$. Three G_q-coupled receptors (5-HT_{2C}, platelet-activating factor, and muscarinic M1 receptors) were transiently expressed in COS-7 cells in the absence or presence of $G\alpha_{14}$. When expressed independently, agonist-induced activation of each receptor led to the stimulation of IP formation (Table 2). Coexpression of $G\alpha_{14}$ with either the 5-HT_{2C} or muscarinic M1 receptors did not enhance the agonist-induced PLCβ response, while a slight enhancement was observed for the platelet-activating factor receptor (Table 2). In contrast, the response elicited by SSTR2 was significantly enhanced in the presence of $G\alpha_{14}$ (Figure 1 and Table 2). $G\alpha_{14}$ seemed to exhibit some selectivity in the recognition of different G_q-coupled receptors.

Lastly, using a $G\alpha_{14}$-specific antiserum, we determined the level of expression of $G\alpha_{14}$ in COS-7 cells transiently coexpressing different GPCRs. Six G_i-coupled receptors were selected; three of them (SSTR2, δ-opioid and the ORL_1 receptors) were functionally linked to $G\alpha_{14}$ whereas the rest (SSTR1, μ- and κ-opioid receptors) were incapable of activating $G\alpha_{14}$ in the preceding experiments. As shown in Figure 6, membranes prepared from COS-7 cells cotransfected with cDNAs encoding $G\alpha_{14}$ and any one of the six receptors exhibited $G\alpha_{14}$-immunoreactivity at equivalent levels. The level of expression of $G\alpha_{14}$ was not affected by the type of GPCR being coexpressed in the cells. The inability of certain GPCRs to activate $G\alpha_{14}$ is therefore unlikely to be due to variations in the expression of $G\alpha_{14}$. It is noteworthy that a weak $G\alpha_{14}$-immunoreactivity was detected in native COS-7 cell membranes. The antiserum for $G\alpha_{14}$ was raised against the carboxyl end (last 10 amino acids) of the polypeptide where the sequence differs from $G\alpha_{q/11}$ by only two residues. This faint signal was probably due to low-level expression of $G\alpha_{14}$ (known to be expressed in the kidney; Nakamura *et al*., 1991) or cross-reactivity with $G\alpha_{q/11}$.

Discussion

The promiscuous coupling of $G_{15/16}$ to a wide spectrum of structurally and functionally distinct GPCRs (Offermanns & Simon, 1995; Lee *et al*., 1998) provided the impetus for us to examine whether G_{14} behaves in a similar fashion. Scattered reports on the coupling of G_{14} to G_i- and G_s-linked receptors (Wu *et al*., 1993; Offermanns & Simon, 1995; Bernhard *et al*., 1996; Maeda *et al*., 1996; Komatsuzaki *et al*., 1997; Yung *et al*., 1998) indicate the promiscuity of G_{14}. Our present study shows that G_{14} is capable of coupling to several more G_i- or G_s-coupled receptors, such as SSTR2, α_2-adrenergic, δ-opioid, and β_2-adrenergic receptors (Tables 1 and 2). Of the

25 different G_i- or G_s-coupled receptors tested in this and previous studies (Wu *et al*., 1993; Offermanns & 1995; Bernhard *et al*., 1996; Maeda *et al*., 1996; Komatsuzaki *et al*., 1997; Yung *et al*., 1998), only eight were capable of interacting with $G\alpha_{14}$. In contrast, $G\alpha_{16}$ has been shown to interact productively with at least 23 GPCRs (Offermanns & Simon, 1995; Wu *et al*., 1995a, b; Zhu & Birnbaumer, 1996; Kuang *et al*., 1996; Lee *et al*., 1998). G_{14} is much less promiscuous than G_{16}.

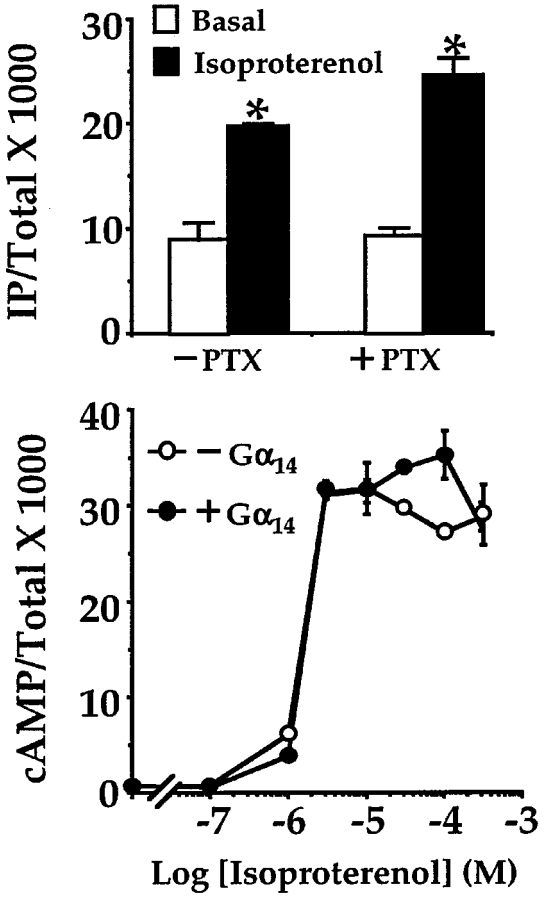

Figure 5 Isoproterenol-dependent stimulation of PLCβ and adenylyl cyclase by the β_2-adrenergic receptor. COS-7 cells were cotransfected with cDNAs encoding the β_2-adrenergic receptor (β_2AR) and $G\alpha_{14}$. For the upper panel, transfected cells were labelled with [^{3}H]-inositol and treated with or without PTX as indicated. IP accumulation was monitored in the absence or presence of 10 μM isoproterenol. *Application of isoproterenol significantly increase IP production; $P<0.05$, paired *t*-test. For the lower panel, transfected cells were labelled with [^{3}H]-adenine and cells pretreated with (solid circles) or without PTX (open circles). Increasing concentrations of isoproterenol (up to 300 μM) was applied to the cells and the corresponding cyclic AMP accumulation was monitored.

The molecular basis by which $G\alpha_{14}$ discriminates receptors is not clearly understood. Recent attempts to define the mechanism by which $G\alpha_{q/11}$ regulates the fidelity of interactions with receptors have identified an N-terminal region which confines the selectivity of receptor coupling (Kostenis *et al*., 1997a). The α subunits of G_q and G_{11} are longer than members of the G_i subfamily by six amino acids (residues 2 to 7), and this insertion appears to prevent $G\alpha_{q/11}$ from coupling to G_i- and G_s-linked receptors. $G\alpha_q$ mutants with one or more of the six residues removed or substituted with alanine are more promiscuous than wild type $G\alpha_q$ in receptor coupling (Kostenis *et al*., 1998). The six-amino acid extension of $G\alpha_{q/11}$ may form a subdomain that is critical for regulating the receptor coupling selectivity of these subunits. However, the relative length of the N-terminal α helix is unlikely to be the sole determinant in receptor coupling selectivity, because the α subunit of the most promiscuous G protein, G_{16}, is actually three residues longer than $G\alpha_{q/11}$ at the N-terminus. Interestingly, $G\alpha_{14}$ is three residues shorter than $G\alpha_{q/11}$ at its N-terminus. Since $G\alpha_{14}$ closely resembles $G\alpha_{q/11}$, in terms of receptor coupling selectivity, it should behave as a mutant of $G\alpha_{q/11}$ with three N-terminal residues deleted. Indeed, both $G\alpha_{14}$ and the three-residue deletion mutant of $G\alpha_{q/11}$ (referred to as qΔ2-4 in Kostenis *et al*., 1998) are capable of interacting with some G_i- and G_s-linked receptors.

Numerous studies have illustrated the importance of the last five amino acids at the C-terminus of the α subunits in determining their selectivity of receptor recognition (Conklin *et al*., 1993; Kostenis *et al*., 1997b). The extreme carboxyl terminus of $G\alpha_{14}$ is almost identical to that of $G\alpha_{q/11}$, with only a phenylalanine substitution for tyrosine at position -4. It remains to be seen if the loss of a hydroxyl group at this location would affect the ability of $G\alpha_{14}$ to recognize receptors. It is noteworthy that $G\alpha_{14}$ is the only one that contains a phenylalanine at position -4, while other α subunits use tyrosine, isoleucine, leucine, or cysteine. Although the two termini of the α subunit are known sites for receptor interaction, other regions may also be involved. Recognition of the chemoattractant complement C5a receptor by G_{16} requires multiple regions of $G\alpha_{16}$ (Lee *et al*., 1995). Perhaps regions other than the two termini may account for the more promiscuous nature of $G\alpha_{14}$ as compared to $G\alpha_{q/11}$.

Recognition of $G\alpha_{14}$ by GPCRs requires the presence of structural motifs on the receptor to be complementary to

Table 2 Coupling of $G\alpha_{14}$ to G_q-linked receptors

		IP formation				
		Without $G\alpha_{14}$		*With $G\alpha_{14}$*		
Receptor	*Ligand*	*Basal*	*Agonist*	*Basal*	*Agonist*	*% Enhancement*
5-HT$_{2c}$	1 μM 5-HT	29.6±3.8	106.1±9.2	22.3±4.9	115.6±11.6	9
Muscarinic M1	200 μM Carbachol	28.3±5.2	228.6±13.3	28.7±5.5	207.4±9.1	0
PAF	100 nM PAF	14.4±2.3	31.6±3.1	14.6±0.6	38.2±1.9	20
SSTR2	100 nM somatostatin	8.7±0.4	25.5±2.3	8.7±0.5	58.1±1.5*	128*

*Agonist-induced IP formation in $G\alpha_{14}$-expressing cells was significantly enhanced over that of the cells without $G\alpha_{14}$; Bonferroni *t*-test, $P<0.05$.

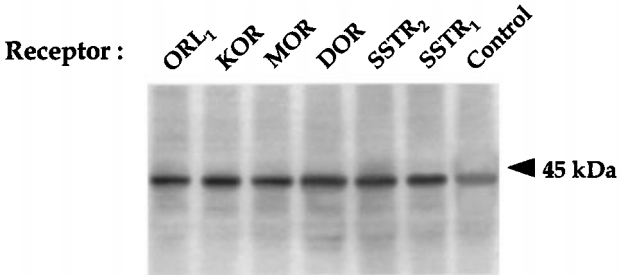

Figure 6 Immunodetection of $G\alpha_{14}$. COS-7 cells were transiently cotransfected with cDNAs encoding $G\alpha_{14}$ and a G_i-coupled receptor as indicated (ORL_1, opioid receptor-like receptor 1; KOR, MOR and DOR, κ-, μ- and δ-opioid receptors; SSTR1 and SSTR2, type 1 and 2 somatostatin receptors). Plasma membranes were prepared 48 h post-transfection. Fifty μg of membrane proteins were separated on a 12.5% gel and electrophoretically transferred to polyvinylidene fluoride membranes. Membranes from untransfected COS-7 cells were included as a control. Protein markers were localized by Ponceau S staining. The expression of $G\alpha_{14}$ was assessed by the $G\alpha_{14}$-specific antisera 3A-195. Two independent experiments with different batches of membrane proteins yielded similar results.

those of $G\alpha_{14}$. In a previous study utilizing deletion mutants of the α_{1B}-adrenergic receptor (Wu *et al*., 1995b), the extreme termini of the third inner loop of the receptor appear critical for interaction with $G\alpha_{14}$. More precisely, a short stretch of amino acids (Tyr-217, Ile-218, and Val-219) of the α_{1B}-adrenergic receptor appears to be required for $G\alpha_{14}$ coupling. However, this motif is not present in the corresponding regions of other GPCRs which are also linked to G_{14} (Table 1). Another motif BBXXB, where B represents a basic residue and X refers to any amino acid, present in the third inner loop of the α_{1B}-adrenergic receptor has been implicated in the coupling to $G\alpha_{14}$ (Wu *et al*., 1995b). The BBXXB motif is found in all of the G_{14}-coupled receptors (Table 1). Yet, the same motif is also found in the third inner loops of GPCRs which are incapable of activating $G\alpha_{14}$ such as the SSTR1, μ- and κ-opioid receptors (Table 1). Alignment of the primary structures of the third inner loops of GPCRs failed to reveal any obvious differences between those receptors which interact with $G\alpha_{14}$ and those that do not. Recently it has been suggested that either one of the two basic amino acids, Lys-158 and Arg-159, present in the second inner loop of the interleukin-8 receptor is required for its coupling to $G\alpha_{16}$ (Xie *et al*., 1997). It is not known if a similar motif is required for GPCRs to interact with $G\alpha_{14}$.

In contrast to earlier reports (Offermanns & Simon, 1995; Wu *et al*., 1995a), the present study shows that the β_2-adrenergic receptor can interact productively with the bovine $G\alpha_{14}$ (Table 1 and Figures 4 and 5). In the report by Wu and co-workers (1995a), the β_2-adrenergic receptor is unable to stimulate PLC activity *via* mouse G_q and G_{14}, but can do so through human G_{16}. Apart from minor experimental details such as the choice of transfection method, one obvious difference is the origin of the $G\alpha_{14}$ cDNAs (mouse versus bovine). However, the primary structures of the mouse and bovine $G\alpha_{14}$ have over 97% amino acid identity, and the minor differences are not located in the putative receptor recognition domains. As we were able to consistently reproduce the coupling between $G\alpha_{14}$ and the β_2-adrenergic receptor, we can only ascribe the difference to variations in experimental setups. With this as a precedent, we therefore cannot entirely exclude the possibility that those GPCRs which were unable to activate $G\alpha_{14}$ in our studies may stimulate $G\alpha_{14}$ under different experimental paradigms.

An interesting twist in the $G\alpha_{14}$ tale is the demonstration that it mediates inhibition of phosphoinositide metabolism in *Xenopus* oocytes (Nakamura *et al*., 1994). The G_q-linked metabotropic glutamate receptor subtype 1 typically stimulates PLCβ in oocytes. Surprisingly, this glutamate response is suppressed in oocytes coexpressing $G\alpha_{14}$. The precise steps and mechanism in receptor activation of G protein functions are not well understood. In this situation there may be different regulatory domains present on the $G\alpha_{14}$ which determines the molecule either to inhibit or stimulate IP formation depending on the type of receptor to which it couples. Another explanation may be that the inhibition of IP metabolism may be due to an unproductive interaction between $G\alpha_{14}$ and metabotropic glutamate receptor subtype 1. The latter is thought to be the more likely mechanism of inhibition (Nakamura *et al*., 1994). In reconstitution experiments, $G\alpha_{14}$, $G\alpha_{11}$ and $G\alpha_q$ are equipotent in coupling the muscarinic m1 receptor to stimulation of PLCβ (Nakamura *et al*., 1995). The same m1 receptor, however, does not appear to use $G\alpha_{14}$ for calcium mobilization in rat basophilic leukaemia cell line RBL-2H3 (Dippel *et al*., 1996). A constitutively active mutant of $G\alpha_{14}$ stimulates PLCβ in a manner similar to cognate mutants of $G\alpha_q$ (Chan *et al*., 2000). Hypothetically, the lack of $G\alpha_{14}$-mediated stimulation of PLCβ may be explained by a simultaneous inhibition of IP formation.

$G\alpha_{14}$ is expressed mainly in the immune system, tissues such as bone marrow stromal cells, spleen, lung and certain myeloid and progenitor B cells (Nakamura *et al*., 1991; Wilkie *et al*., 1991). The expression patterns in the mouse tissues suggest that $G\alpha_{14}$ may be involved in the regulation of signal transduction pathways of cellular immunity. Interestingly, all the G_{14}-coupled receptors identified in this study are expressed in various types of inflammatory cells and they are involved in inflammatory responses. δ-opioid receptor-specific ligands have been demonstrated to mediate immunosuppressive effects (House *et al*., 1995; Roy & Loh, 1996). Application of adrenergic receptor agonists relieved asthma symptoms, inhibited the secretion of bronchoconstrictor mediators from airway mast cells and altered the production of tumour necrosis factor-α, interleukin-6, -10, and -12, macrophage inflammatory protein 1α and the production of nitric oxide (Hasko & Szabo, 1998; Barnes, 1999). SSTR2 regulates the release of interferon-γ in T cells (Elliott *et al*., 1999). Furthermore, somatostatin activated the IP production through SSTR2 but not SSTR1 in pituitary carcinoma F_4C_1 cells and the response was only partially sensitive to PTX (Chen *et al*., 1997). G_{14} may serve as a convergent point for extracellular signals in immune cells and mediates Ca^{2+} mobilization, which is known to be critical for triggering inflammatory responses.

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