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Differential Coupling of Gαq Family of G-protein to Muscarinic M_1 Receptor and Neurokinin-2 Receptor

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The ligand binding signals to a wide variety of seven transmembrane cell surface receptors are transduced into intracellular signals through heterotrimeric G-proteins. Recently, there have been reports which show diverse coupling patterns of ligand-activated receptors to the members of Gq family α subunits. In order to shed some light on these complex signal processing networks, interactions between Gαq family of G protein and neurokinin-2 receptor as well as muscarinic M_1 receptor, which are considered to be new thearpeutic targets in asthma, were studied. Using washed membranes from Cos-7 cells co-transfected with different Gαq and receptor cDNAs, the receptors were stimulated with various concentrations of carbachol and neurokinin A and the agonist-dependent release of [^{3}H]inositol phosphates through phospholipase C beta-1 activation was measured. Differential coupling of Gαq family of G-protein to muscarinic M_1 receptor and neurokinin-2 receptor was observed. The neurokinin-2 receptor shows a ligand-mediated response in membranes co-transfected with Gαq, Gα11 and Gα14 but not Gα16 and the ability of the muscarinic M_1 receptor to activate phospholipase C through Gαq/11 but not Gα14 and Gα16 was demonstrated. Clearly Gαq/11 can couple M1 and neurokinin-2 receptor to activate phospholipase C. But, there are differences in the relative coupling of the Gα14 and Gα16 subunits to these receptors.

Key words: Muscarinic receptor, Neurokinin-2 receptor, Gαq G-protein, Phospholipase C beta, Signal transduction. Phosphoinositide

INTRODUCTION

Heterotrimeric G-Proteins transduce ligand binding to a wide variety of seven transmembrane cell surface receptors into intracellular signals (Gudermann *et al.*, 1996). Seventeen different isoforms of the Gα subunit protein have been identified and classified into four families, Gαs, Gαi, Gαq and Gα12 according to sequence homologies (Simon *et al.*, 1991). It has been reported that the receptors upon activation generating the intracellular cAMP preferentially couple to the members of Gs family (Gilman, 1987), whereas the receptors which induce pertussis toxin-insensitive activation of phospholipase C are mainly linked to the members of the Gαq family (Berridge, 1993). The Gq family consists of five members whose α-subunits show different expression patterns. Gαq and Gα11 seem to be almost ubiquitously expressed, whereas Gα14 is predominantly expressed in spleen, lung, kidney and testis (Wilkie *et al.*, 1991). Gα16 is expressed in a hematopoietic cells (Amatruda, *et al.*, 1991). Beta-isoforms of phospholipase C were shown to be activated by all members of Gαq family, i.e., Gαq, Gα11, Gα14 and Gα16 subunits either in purified form or in membrane-enriched form in a reconstitution system (Rhee and Bae, 1997). The muscarinic M_1 receptor and neurokinin-2 receptor, which are considered to be new therapeutic targets in asthma, mediate the biological effects of acetylcholine and neurokinin A, respectively (Zaagsma *et al.*, 1997 and Longmore *et al.*, 1995). The Muscarinic M_1 receptor subtype has been shown to be involved in the control of airway smooth muscle diameter of various species including man (Maclagan and Barnes, 1989; Barnes, 1990a; Barnes, 1993b). Within the respiratory system, tachykinins have a number of important physiological effects such as bronchoconstriction of large airways, enhancement of vascular permeability and stimulation of mucus secretion (Naline *et al.*, 1989). Among three tachykinin receptor families, neurokinin-2 receptor, which is selective toward neurokinin A, predominates in animal and human airways. In experiments using cDNA transfection with intact cells, all four Gq family α subunits were found to stimulate phospho-

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lipase C β_1-activated inositol phosphate release from Cos-7 cells (Wu *et al.*, 1992a and 1992b). Recently, there have been reports which show diverse coupling patterns of ligand-activated receptors to the members of Gq family α subunits (Gudermann, *et al.*, 1997). In order to shed some light on these complex signal processing network, interactions between Gαq family of G protein and neurokinin-2 receptor as well as muscarinic M_1 receptor were studied. In a previous report, washed membranes from cells transfected with different Gαq cDNAs and enriched for specific Gα G proteins were used to measure GTPγS-mediated activation of purified phospholipase C (Lee *et al.*, 1992 and Wu *et al.*, 1992). With the same system we report the functional coupling between members of Gαq subunits and the muscarinic M_1 and neurokinin-2 receptors.

MATERIALS AND METHODS

Materials were obtained from the following sources: Carbachol and neurokinin A are from Research Biochemical International (Natick, MA, USA); GTPγS from Boehringer-Mannheim (Mannheim, Germany); [^{3}H]phosphatidylinositol 4,5-bisphosphate from Amersham Buckinghamshire, England); Dulbecco's modified Eagle medium, fetal calf serum and 100 mm culture plates are from Gibco-BRL (Gaithersburg, MD, USA); Lipofectin and opti-MEM from Bethesda Research Laboratories (Bethesda, USA); All of the receptor and Gα subunits cDNAs were kindly provided by Mel. I. Simon (Caltech, USA); Purified phospholipase C β_1 was provided by Sue Goo Rhee (NIH, Bethesda, USA); Other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Cos-7 cell expression vector

cDNAs corresponding to G-protein α-subunits Gαq, Gα11, Gα14, Gα16, muscarinic M_1 and neurokinin-2 receptor were carried by the cytomegalovirus vector pCMV.

Transient transfection of Cos-7 cells

Cos-7 cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Cells were seeded at a density of 2.5×10^6/plate were in 100 mm plates a day before transfection. In cotransfection with two different plasmids, 5 μg of each plasmid was mixed with 80 μl of lipofectin in 6 ml of opti-MEM and was added to the cells. In control experiments, the total amount of cDNA was maintained constant by adding pCMV DNA. Fetal calf serum (20%) in 6 ml of Dulbecco's modified Eagle medium was added after 5 h. After 2 days the cells were collected for the membrane preparation.

Preparation of receptor and Gα subunit-cotransfected Cos-7 cell membranes

Various receptors and Gα subunit-cotransfected Cos-7 cell membranes were prepared as described previously (Lee *et al.*, 1992). Three to five plates of cells cotransfected with each of receptor and Gα subunit (i. e., muscarinic M_1 receptor, muscarinic M_1 receptor+Gα11, muscarinic M_1 receptor+Gαq, muscarinic M_1 receptor+Gα14, muscarinic M_1 receptor+Gα16, neurokinin-2 receptor, neurokinin-2 receptor+Gα11, neurokinin-2 receptor+Gαq, neurokinin-2 receptor+Gα14 and neurokinin-2 receptor+Gα16) were washed with cold phosphate-buffered saline, followed by scraping in the presence of homogenization buffer (50 mM HEPES, 0.2 mM EGTA, pH 7.0, 0.01% soybean trypsin inhibitor, 1 mM phenylmethylsufonyl fluoride, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A and 1 mM dithiothreitol). Cell suspensions were homogenized in 3~4 ml of buffer, followed by centrifugation at 500× g for 5 min. The supernatant was further spun down at 100,000×g for 40 min. The pellets were then resuspended and wahed with 0.8~1.0 ml of cold extraction buffer (1 M KCl in 0.5×homogenization buffer) for 2~3 h. The final washed membrane pellets were resuspended in 400 μl of homogenization buffer and aliquots were frozen in liquid nitrogen for storage at -70°C until use.

Agonist-induced receptor activation assay

The assay mixture consisted of 30 μl of assay buffer (50 mM HEPES, pH 7.0, 100 mM KCl, 6 mM $MgCl_2$, 0.6 mM $CaCl_2$, 2 mM EGTA), 15~20 μM PIP_2 (12,000~15,000 cpm of [^{3}H]PIP_2), five units of phospholipase C β_1, GTPγS and membrane preparations cotransfected with various receptors and Gα subunits (final volume of 50 μl). The amounts of enzyme used for each assay were adjusted to the same level in terms of enzyme activity units, which were defined by measuring the Ca^{2+} stimulated activity at 4 μM Ca^{2+}. One unit of enzyme activity was defined as 10 μmol of PIP_2 hydrolysis/min/mg of protein. The receptors were stimulated with various concentrations of carbachol or neurokinin A and the agonist-induced release of [^{3}H]inositol phosphate was measured as described previously (Lee *et al.*, 1992).

GTPγS-induced G-protein activation assay

The assay mixture consisted of 30 μl of assay buffer (50 mM HEPES, pH 7.0, 100 mM KCl, 6 mM $MgCl_2$, 0.6 mM $CaCl_2$, 2 mM EGTA), 15~20 μM PIP_2 (12,000~15,000 cpm of [^{3}H]PIP_2), five units of phospholipase C β_1 and membrane preparations cotransfected with various receptors and Gα subunits (final volume of 50 μl). The assay mixture was incubated

on ice for 10 min and the reaction was started by the addition of GTPγS (1 μM final). After 15 min incubation at 37°C, the reaction was terminated and the GTPγS-dependent release of [^{3}H]inositol phosphate was measured as described (Lee *et al.*, 1992).

Estimation of the amounts of Gα subunit protein in the receptor and Gα subunit-cotransfected Cos-7 cell membranes

The amounts of Gα11, Gαq, Gα14 and Gα16 protein in the membrane preparation was estimated as follows; in brief, specific antipeptide antisera were used and Gα11 protein for constructing standard curves was obtained by purification from recombinant protein made in *Escherichia coli* (Amatruda *et al.*, 1991). The amount of each Gα subunits was adjusted to the same level as that of Gα11 by comparison with measured level as that of Gα11 using an antibody prepared against the internal amino acid sequence (GESGKSTFIKQMRIIHG), which is common in Gα11, Gαq, Gα14 and Gα16 proteins. The level of G-protein used in each assay was adjusted to be in the order of 10 ng which corresponded to 0.8~1.0 mg of total membrane.

RESULTS AND DISCUSSION

Previous pharmacological studies indicated that muscarinic M_1 receptor evoked phosphoinositide hydrolysis response in many tissue preparations (Ford *et al.*, 1992; Day *et al.*, 1991; Hulme *et al.*, 1990) and three tachykinin receptors were characterized to be coupled to Ca^{2+} signaling pathways in many experimental preparations, which is assumed to be mediated by agonist-induced phosphoinositide hydrolysis (Hellstrom *et al.*, 1994; Nakajima *et al.*, 1992). These tachykinin receptors were also shown to modulate the activity of adenylyl cyclase through coupling to Gs protein in other experiment systems (Laniyonu *et al.*, 1988; Yamashita *et al.*, 1983; Hunter *et al.*, 1985). However, the precise characterization of intracellular signal transduction involved in each of the receptors has not been fully elucidated, especially in terms of specific coupling with each member of α-subunit of Gq family of G-protein. A cell-free membrane reconstitution system was used to study the relative specificity of interaction between different receptors and G-proteins of the Gαq family. cDNA clones encoding receptors and G-protein α-subunits were transiently cotransfected into Cos-7 cells in which members of the Gαq sub-family and receptors could be overproduced. The relative ability of the different Gα subunit-enriched membrane preparations to activate PI-PLC β_1 was tested. As shown in Fig. 1, all four Gα subunits (Gαq, Gα11, Gα14 and Gα16) showed more

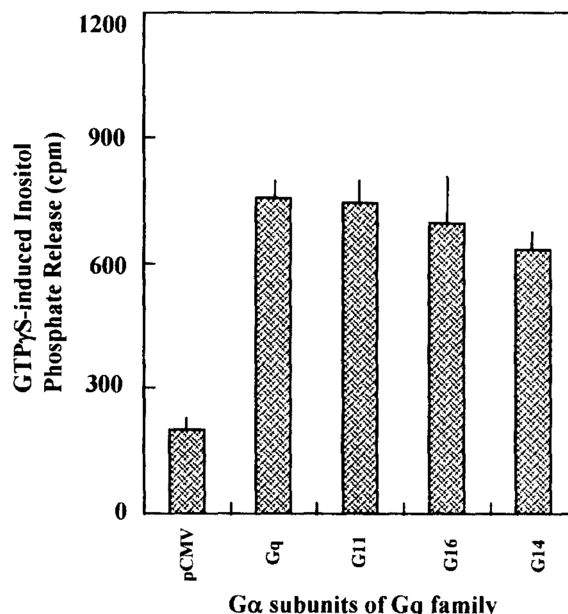

Fig. 1. Relative abilities of the different Gα subunit-enriched membrane preparations to activate phosphatidylinositol-specific phospholipase $C\beta_1$. Various Gα subunit-transfected cos-7 cell membranes were prepared as described under "Materials and Methods". The membranes were incubated in the presence of 5 units of phospholipase $C\beta_1$ and GTPγS-induced [^{3}H]inositol phosphates release was measured as described under "Materials and Methods". Data shown are mean values of two to three duplicate observations and the variation is less than 10%.

or less similar level of GTPγS-dependent activation of PIP_2 hydrolysis by PI-PLC β_1 with membranes prepared from cells cotransfected with muscarinic M_1 or neurokinin-2 receptor and α-subunits of Gαq family. There were approximately 3~4 folds increases in the level of GTPγS-induced inositol phosphate accumulation as compared with that of the control (in the absence of GTPγS). Similar results were observed in experiments using cDNA transfection with intact cells, where all four Gαq family α-subunits were found to stimulate PI-PLC β_1-activated inositol phosphate release from Cos-7 cells (Lee *et al.*, 1992 and Wu *et al.*, 1992a, b). Fig. 2 shows that muscarinic M_1 receptor-induced inositol phosphate production in response to carbachol in washed membranes from cells transfected with different Gαq subunits and muscarinic M_1 receptor cDNA that were enriched for specific Gα subunits and receptor proteins. Membrane preparations from cotransfected with muscarinic M_1 receptor cDNA and cDNA encoding Gαq and Gα11 subunit showed an increase in carbachol concentration-dependent formation of inositol phosphate with similar efficacy. However, membrane preparations from cotransfected with muscarinic M_1 receptor cDNA and cDNAs encoding Gα14 or Gα16 subunits showed little or no increase in ligand-induced formation of inositol phos-

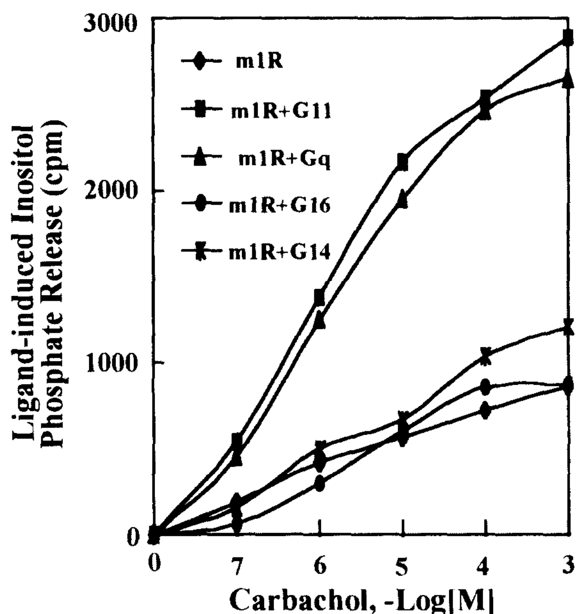

Fig. 2. Muscarinic M_1 receptor-induced inositol phosphate production in response to various concentrations of carbachol. Washed membranes from cells cotransfected with muscarinic M_1 receptor and various Gα subunits cDNAs which were enriched for Gα and receptor proteins were prepared and carbachol-dependent [^{3}H]inositol phosphates release was measured as described under "Materials and Methods". Data shown are mean values of two to three duplicate observations and the variation is less than 10%.

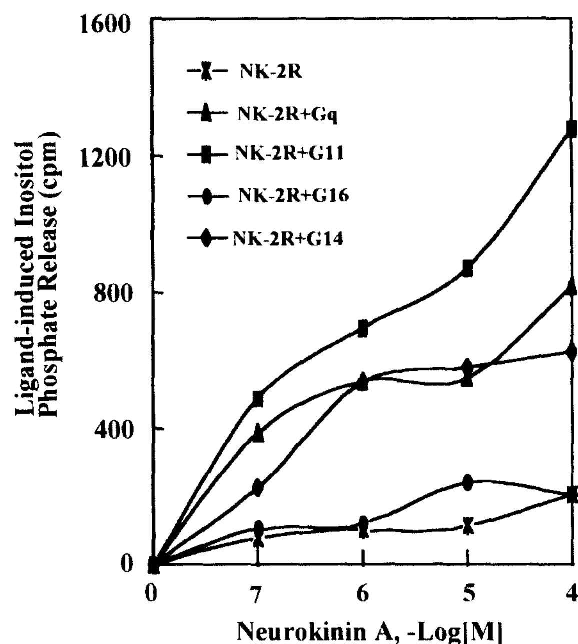

Fig. 3. Neurokinin-2 receptor-mediated inositol phosphate formation in membranes from cells transfected with cDNAs corresponding different Gαq class subunits and neurokinin-2 receptor cDNA. Membrane preparations cotransfected with neurokinin-2 receptor and various Gα subunits were prepared and neurokinin-2-dependent [^{3}H]inositol phosphate release was measured as described under "Materials and Methods". Data shown are mean values of two to three duplicate observations and the variations are less than 10%.

phates over those transfected with muscarinic M_1 receptor cDNA alone. Although there have been sporadic reports which show the specific interaction between muscarinic M_1 receptor and Gαq/Gα11 subunits in different experimental conditions (Ross and Berstein, 1993; Brauner-Osborne and Brann, 1996; Bernstein *et al.*, 1992), there has been no report which elucidates the overall coupling characteristics between all the α-subnit of Gq family of G protein and muscarinic M_1 receptor in the same experimental system which includes the necessary transmembrane signal transduction components such as receptor, Gα subunit of G-protein and effector. Here our data clearly shows the ability of the muscarinic M_1 receptor to activate phospholipase C β_1 through Gαq/11, but Gα14 and Gα16 shows little or no interaction with muscarinic M_1 receptor. Therefore, muscarinic M_1 receptor subtype-mediated physiological functions, for example airway smooth muscle control and others, may be performed mainly through Gαq/11 but not through Gα14 and Gα16. Although the coupling ability of neurokinin-1 receptor to the Gαq/Gα11 subset of Gαq family was demonstrated through the ligand-binding study in the purified, reconstitution system (Kwatra *et al.*, 1993), coupling abilities of the rest of the tachykinin receptor family have not been reported. In contrast with the muscarinic M_1 receptor, the neurokinin-2 receptor shows somewhat different coupling pattern with α-subunits of Gq family of G-protein. As shown in Fig. 3, samples from cells co-transfected with neurokinin-2 receptor cDNA and cDNA encoding Gαq, Gα11 and Gα14 subunit showed different levels of increase in neurokinin A-induced formation of inositol phosphate in dose-dependent manner, especially with maximum efficacy in Gαq-transfected membrane. Neurokinin-2 receptor showed more or less same efficacy of interaction with Gα11 and Gα14 subunit, respectively. In addition, cell membrane preparations from cotransfected with neurokinin-2 receptor cDNA and cDNAs encoding Gα16 subunits showed no increase in ligand-dependent formation of inositol phosphates over those transfected with receptor cDNA alone. However, it has been reported that Gα16 can be activated by a wide variety of G-protein coupled receptors, such as $5HT_{1A}$, $5HT_{1C/2C}$, thromboxane TXA_2, fMLP, β_2-adrenergic, dopamine D_1, muscarinic M_2, vasopressin V_2, vasopressin V_{1A}, adenosine A_{2A} and thrombin receptors (Offermanns and Simon, 1995). Therefore, even if Gα16 seems to be considered to have promiscuous interaction with many kinds of G-protein coupled receptors, this behaviour does not hold true in cases of the muscarinic M_1 and neurokinin-2 receptors. Taken together, Gαq and Gα11 can couple

to muscarinic M_1 and neurokinin-2 receptor and Gα 14 couples to neurokinin-2 receptor, not to muscarinic M_1 receptor to activate phospholipase C β_1.

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