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G protein subunits and the stimulation of phospholipase C by G_s- and G_i-coupled receptors: Lack of receptor selectivity of $G\alpha_{16}$ and evidence for a synergic interaction between $G\beta\gamma$ and the α subunit of a receptor-activated G protein

(receptor–effector coupling/luteinizing hormone receptor/V2 receptor/β-adrenergic receptors/signal transduction)

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ABSTRACT **Stimulatory guanine nucleotide binding protein (G_s)-coupled receptors activated by luteinizing hormone, vasopressin, and the catecholamine isoproterenol (luteinizing hormone receptor, type 2 vasopressin receptor, and types 1 and 2 β-adrenergic receptors) and the G_i-coupled M2 muscarinic receptor (M2R) were expressed transiently in COS cells, alone and in combination with $G\beta\gamma$ dimers, their corresponding $G\alpha$s ($G\alpha_s$ or $G\alpha_{i3}$) and either $G\alpha_q$ or $G\alpha_{16}$. Phospholipase C (PLC) activity, assessed by inositol phosphate production from preincorporated *myo*[^{3}H]inositol, was then determined to gain insight into differential coupling preferences among receptors and G proteins. The following were observed: (*i*) All receptors tested were able to stimulate PLC activity in response to agonist occupation. The effect of the M2R was pertussis toxin sensitive. (*ii*) While, as expected, expression of $G\alpha_q$ facilitated an agonist-induced activation of PLC that varied widely from receptor to receptor (400% with type 2 vasopressin receptor and only 30% with M2R), expression of $G\alpha_{16}$ facilitated about equally well the activation of PLC by any of the tested receptors and thus showed little if any discrimination for one receptor over another. (*iii*) $G\beta\gamma$ elevated basal (agonist independent) PLC activity between 2- and 4-fold, confirming the proven ability of $G\beta\gamma$ to stimulate PLCβ. (*iv*) Activation of expressed receptors by their respective ligands in cells coexpressing excess $G\beta\gamma$ elicited agonist stimulated PLC activities, which, in the case of the M2R, was not blocked by pertussis toxin (PTX), suggesting mediation by a PTX-insensitive PLC-stimulating $G\alpha$ subunit, presumably, but not necessarily, of the G_q family. (*v*) The effects of $G\beta\gamma$ and the PTX-insensitive $G\alpha$ elicited by M2R were synergistic, suggesting the possibility that one or more forms of PLC are under conditional or dual regulation of G protein subunits such that stimulation by one sensitizes to the stimulation by the other.**

Many cell surface receptors respond to the occupation by hormones and neurotransmitters by activating heterotrimeric guanine nucleotide binding proteins (G proteins). The activation of a G protein is accomplished by the dissociation of its α subunit from the $\beta\gamma$ dimer, both of which in turn modulate target effectors. Conventional classification defines a given G-protein-coupled receptor according to the $G\alpha$ subunit involved in the coupling event. This is usually determined by the primary function the receptor has on the target effectors. Thus, G_s-coupled receptors stimulate adenylyl cyclase, G_i-coupled receptors inhibit adenylyl cyclase, and G_q-coupled receptors stimulate phospholipase C (PLC) (1). Physiologically, it is very common that an extracellular stimulus can activate multiple signaling pathways through multiple receptor subtypes coupling to different G proteins. However, this may not be true in all cases in which multiple signaling events occur in response to stimulation by a single agonist. Several hormones are known to modulate multiple effectors without apparently having multiple receptors (2–4). Indeed, a large number of G-protein-coupled receptors have been shown to induce multiple signaling pathways when expressed in model host cells (4–6).

Previously, by studying several structurally unrelated G_s-coupled receptors stably expressed in murine fibroblast L cells at different levels, we found that they all shared the ability to stimulate PLC in addition to stimulating adenylyl cyclase. PLC stimulation was dependent on a higher level of receptor expression than adenylyl cyclase stimulation (2–4, 7). Dual signaling of this type could result from receptors interacting with multiple G proteins, which in this case would be G_s for stimulation of adenylyl cyclase and G_q, or other members of the G_q family, for stimulation of PLC. On the other hand, a large body of evidence has now shown that the free G-protein $\beta\gamma$ dimers also play an important role in signal transduction processes (8). It is thus well known that the activities of at least two PLC subtypes, PLCβ$_2$ and PLCβ$_3$, are stimulated by $G\beta\gamma$ (9–13). In fact, it is widely accepted that $G\beta\gamma$ is the signal transducer for the pertussis toxin (PTX)-sensitive PLC stimulation found for many G_i-coupled receptors (9). A similar mechanism could also be at play when G_s is activated, providing an alternative explanation for dual signaling induced by G_s-coupled receptors. In this study, we investigated the abilities of the α subunits of G_q and G_{16} (members of G_q family) and the $G\beta\gamma$ dimer to facilitate stimulation of PLC by G_s-coupled receptors using COS-M6 cells transiently cotransfected with the desired G-protein subunits and the receptors used in our previous study (7)—i.e., the murine luteinizing hormone receptor (LHR), the human V2 vasopressin receptor (V2R) and the human β1 and β2 adrenergic receptors (β1AR and β2AR). In addition, the type 2 muscarinic receptor (M2R) was included as an example of a G_i-coupled receptor known to signal to PLC through $G\beta\gamma$. We found that while $G\alpha_q$-mediated PLC activation displayed receptor specificity, $G\alpha_{16}$ promoted ligand-stimulated PLC activation for all the receptors tested, regardless of their presumed G-protein specificity. Overexpression of $G\beta\gamma$ dimers not only increased basal PLC

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Abbreviations: AR, adrenergic receptor; β1AR, type 1 βAR; β2AR, type 2 βAR; G protein, guanine nucleotide binding protein; LHR, luteinizing hormone receptor; M2R, type 2 muscarinic receptor; PLC, phosphatidylinositol-specific phospholipase C; PTX, pertussis toxin; V2R, type 2 vasopressin receptor.

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activity but also facilitated agonist-stimulated PLC activation by all the receptors.§

MATERIALS AND METHODS

Materials. Human chorionic gonadotropin, arginine vasopressin, (−)-isoproterenol, carbachol, and chloroquine were purchased from Sigma. DEAE-dextran was from Pharmacia. PTX was obtained from List Biological Laboratories (Campbell, CA). *Myo*-[^{3}H]inositol (20 Ci/mmol; 1 Ci = 37 GBq) was from American Radiolabeled Chemicals (St. Louis). All tissue culture reagents were purchased from GIBCO.

cDNAs and Expression Vectors. cDNAs for receptors LHR, V2R, β1AR, β2AR, M2R, and the Gα-protein subunits $G\alpha_s$, $G\alpha_{i3}$, $G\alpha_q$, $G\alpha_{16}$, and $G\beta_1$ were subcloned into the eukaryotic expression vector pKNH (15). cDNAs for $PLC\beta_1$, $PLC\beta_2$, and $G\gamma_2$ were subcloned into the pMT2 expression vector (16). A β-galactosidase gene from *Escherichia coli* was subcloned into pKNH and used as a supplementary cDNA in transfection mixtures and to monitor transfection efficiency.

Transient Transfection of COS-M6 Cells. COS-M6 cells were maintained under subconfluent conditions at 5% CO_2/95% air in Dulbecco's minimum essential medium containing 4.5 mg of D-glucose per ml, 10% heat-inactivated fetal bovine serum, 50 units of penicillin per ml, and 50 μg of streptomycin per ml at 37°C. Transient transfection was performed according to Sambrook *et al.* (16) with modifications. Briefly, cells were seeded in 12-well plates at a density of 250,000 cells per well 16 hr before transfection. A transfection mixture was made with DEAE-dextran at 0.5 mg/ml in Tris-buffered saline containing 0.1% D-(+)-glucose (TBS-D) and the plasmid DNA. The total amount of DNA used was kept at 0.5 μg per 100 μl of the DEAE-dextran solution. This is achieved by mixing 0.1 μg of each plasmid containing the desired cDNA and a variable amount of control vector, β-galactosidase in pKNH, to make to a total of 0.5 μg of plasmid DNA. After washing with Hanks' balanced salt solution (HBSS) and TBS-D, the cells were treated with 100 μl of the transfection mixture at room temperature for 15 min followed by addition of 400 μl of a DMEM solution containing 125 μM chloroquine, 2.5% fetal bovine serum, and 10 mM Hepes buffer at pH 7.4. The plates were incubated at 37°C for 1–2 hr and the cells were treated with 10% dimethyl sulfoxide (DMSO) in HBSS for 2 min followed by two washes with serum-free DMEM. Then, 1 ml of *myo*-inositol-free DMEM containing 2 μCi of *myo*-[^{3}H]inositol and 10% fetal bovine serum was added to each well and cells were incubated at 37°C for 40 hr. The transfection efficiency was usually ≈50% as determined by staining cells transfected with β-galactosidase in pKNH with X-Gal as described by MacGregor *et al.* (17).

PLC Activity. Basal and receptor stimulated PLC activities were assessed by the measurement of the accumulation of inositol phosphates as described (7). The levels of *myo*-[^{3}H]inositol and [^{3}H]inositol phosphates were determined by scintillation counting.

Data Presentation. Data, expressed as the percentage of [^{3}H]inositol phosphates in the sum of *myo*[^{3}H]inositol plus [^{3}H]inositol phosphates (7), are averages ± SD of three transfection experiments.

RESULTS

To establish the role of each G-protein subunit in PLC activation, we measured under basal conditions—i.e., omitting addition of receptor ligands, the accumulation of inositol phosphates in COS-M6 cells transfected with different G-protein subunits. Compared to cells transfected with β-galactosidase alone, basal PLC activity was 50% and 30% higher in cells transfected with $G\alpha_q$ and $G\alpha_{16}$, respectively, whereas the activity in cells transfected with $G\alpha_s$ or $G\alpha_{i3}$ was not significantly different from that in control cells. Expression of $G\beta_1\gamma_2$ dimer caused a dramatic increase in basal PLC activity, nearly 3-fold, and this increase was partially blocked by coexpression with $G\alpha_s$ or $G\alpha_{i3}$ (Fig. 1).

It has been shown that $PLC\beta_2$ can be stimulated by $G\beta\gamma$, whereas $PLC\beta_1$ cannot (9, 10). In the present study, expression of either $PLC\beta_1$ or $PLC\beta_2$ resulted in an ≈2-fold increase in basal accumulation of inositol phosphates. Coexpression of $G\beta_1\gamma_2$ and $PLC\beta_1$ did not cause a further elevation of inositol phosphate accumulation, whereas $G\beta_1\gamma_2$ and $PLC\beta_2$ caused a marked elevation in PLC activity (Fig. 1). The effect of $G\beta_1\gamma_2$ was prevented when the wild-type $G\gamma_2$ was substituted with a nonprenylated mutant form of $G\gamma_2$ in which the cysteine residue at position 68 was mutated to a serine (data not shown).

Coexpression of $G\alpha_q$ or $G\alpha_{16}$ with $PLC\beta_2$ resulted in an increase of inositol phosphate accumulation under basal conditions. $G\alpha_{16}$ and $G\beta_1\gamma_2$ apparently have a synergistic effect on endogenous PLC activity, in agreement with the previous finding of Wu *et al.* (13). Addition of $PLC\beta_2$ to the system did not increase further the accumulation of inositol phosphates (Fig. 1).

Transfection of receptor cDNA into COS cells resulted in densities, determined by whole cell radioligand binding assays (7), that ranged from 200,000 to 4,000,000 per cell for all the receptors studied—i.e., LHR, V2R, β1AR, β2AR, and M2R. When transfected alone, all receptors stimulated PLC activity in COS cells from 38% to 350%, acting with a rank order of efficacy of V2R > LHR > β1AR > M2R > β2AR (Fig. 2).

One possibility as to why these receptors stimulate PLC is that they activate directly PLC-stimulating G proteins, such as G_q, G_{11}, G_{14}, or G_{16}. Basal PLC activity increased only slightly when rat $G\alpha_q$ or human $G\alpha_{16}$ was coexpressed with receptors. In contrast, the ligand-stimulated PLC activities were increased by ≈6-fold over basal for all the receptors tested when they were coexpressed with $G\alpha_{16}$ but varied with receptor type when coexpressed with $G\alpha_q$. Thus, while for V2R and LHR, coexpression of $G\alpha_q$ increased the ligand-stimulated activity to an extent close to that obtained by coexpression with $G\alpha_{16}$, the effect of $G\alpha_q$ was less with the β1AR, very weak with the β2AR, and undetectable with the M2R (Fig. 2).

The second possible reason for the dual coupling capability of the G-protein-coupled receptors is that the secondary pathway could be mediated by free βγ subunits, which are

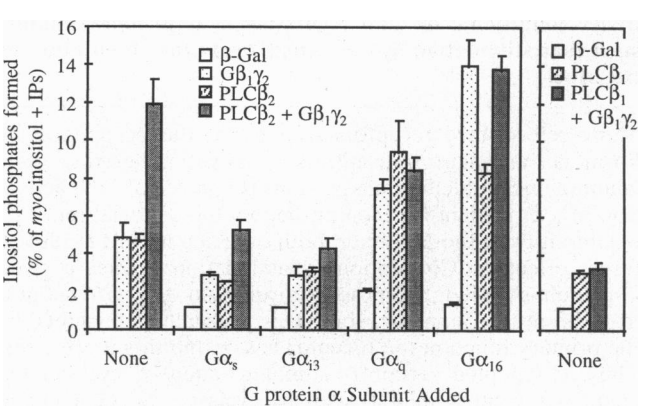

FIG. 1. Effects of G-protein α subunits on increased intrinsic activity of inositol phosphate accumulation caused by G-protein βγ dimer and $PLC\beta_2$. COS-M6 cells were transfected with plasmids containing cDNAs as indicated and prelabeled with *myo*[^{3}H]inositol for 40 hr. Accumulation of [^{3}H]inositol phosphates under the basal condition was determined as described.

§A preliminary report of these findings was presented at the 77th Annual Meeting of the Endocrine Society in Washington, DC (June 14–17, 1995) (14).

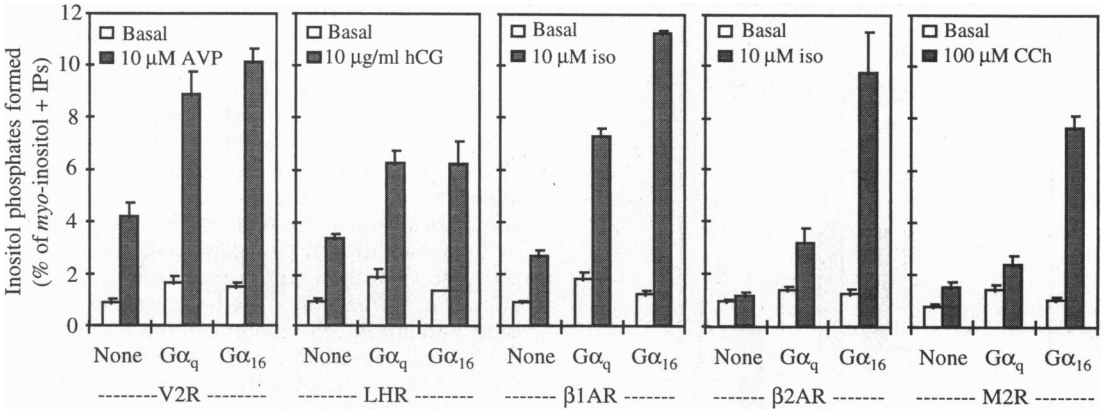

FIG. 2. Effect of $G\alpha_q$ and $G\alpha_{16}$ on coupling of human V2R, murine LHR, human β1AR and β2AR, and M2R to PLC activity. COS-M6 cells were transfected with plasmids containing cDNAs as indicated and prelabeled with *myo*[^{3}H]inositol for 40 hr. Accumulation of [^{3}H]inositol phosphates under basal and stimulated conditions was determined as described. AVP, arginine vasopressin; hCG, human chorionic gonadotropin; iso, isopreterolol; CCh, carbachol.

generated upon dissociation of the heterotrimer during activation. Expression of $G\beta\gamma$ ($\beta_1\gamma_2$) markedly augmented intrinsic PLC activity and activation of the receptors allowed for further stimulation of PLC up to 2-fold (Fig. 3). Coexpression of $G\alpha_s$ with $G\beta\gamma$ partially prevented the rise of the basal activity as well as ligand-stimulated PLC activities for the G_s-coupled receptors. This effect was mimicked by $G\alpha_{i3}$ for the M2R (Fig. 3). PLCβ_2 is known to be activated by free $G\beta\gamma$ subunits (9–12). Coexpression of $G\beta\gamma$ with PLCβ2 raised the basal level of inositol phosphate production (Fig. 1). Interestingly, even though agonists stimulated PLC activity in cells expressing G_s- or G_i-coupled receptors and $G\beta\gamma$, and agonist-independent (basal) PLC activity was increased by expression PLCβ2 in receptor plus $G\beta\gamma$-expressing cells, we found that agonist-stimulated PLC activity was not higher in receptor, $G\beta\gamma$ plus PLCβ2 expressing cells than in cells expressing only receptor plus $G\beta\gamma$, no matter whether $G\alpha_s$ (or $G\alpha_{i3}$ for M2R) was also coexpressed (data not shown).

PTX uncouples G_i proteins through ADP-ribosylation of their α subunits. In cells transfected with M2R, PTX treatment abolished carbachol-stimulated PLC activity by 60% and 40%, respectively, when the receptor was expressed alone or together with the complete G_{i3} protein (i.e., $G\alpha_{i3}\beta_1\gamma_2$). However, if only free $G\beta\gamma$ subunits were coexpressed with M2R, the carbachol-stimulated activity was inhibited <20% by PTX treatment—i.e., largely PTX insensitive.

DISCUSSION

Using overexpression of G-protein subunits of two β-type PLCs and of several G-protein-coupled receptors in COS cells, we confirmed previous findings that PLCβ2, but not PLCβ1, is stimulated by $G\beta\gamma$ and that $G\alpha_q$ and $G\alpha_{16}$, but not $G\alpha_s$ and $G\alpha_i$, possess PLC-stimulating activity (9, 12, 13). The failure of $G_s\alpha$ and $G_i\alpha$ to stimulate PLC was evidenced from the fact that rather than enhancing or leaving unaffected $G\beta\gamma$-stimulated PLC activity, $G\alpha_s$ and $G\alpha_i$ decreased the $G\beta\gamma$-stimulated activity, acting most likely as scavengers of $G\beta\gamma$. In addition, four aspects of the results obtained in this study were unexpected and of interest.

First, as reported earlier in preliminary form (14), we discovered that $G\alpha_{16}$ interacted with all the receptors tested and exhibited only minimal, if any, selectivity. Independent studies by Offermanns and Simon (18) agree with this observation. It had been speculated that under the conditions of expression used here, $G\alpha_{16}$ remained for the most part free—i.e., unassociated to $G\beta\gamma$ (13). However, the fact that receptors promoted $G\alpha_{16}$-mediated activation of PLC indicates the formation of functionally active heterotrimers. Receptors are known to interact and promote activation of α subunits only in the context of their association with $\beta\gamma$ dimers.

Second, we found that classical G_s-coupled receptors have the ability to stimulate PLC activity through the ubiquitously expressed G_q but that, in contrast to their interaction with G_{16},

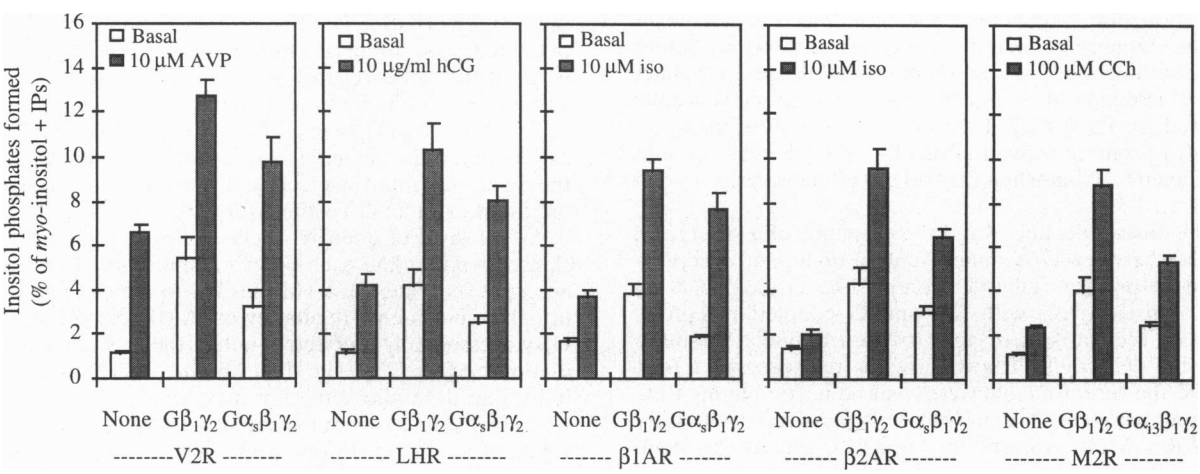

FIG. 3. Effect of G-protein $\beta\gamma$ subunits on coupling of the G_s- or G_i-coupled receptor to PLC activity. COS-M6 cells were transfected with plasmids containing cDNAs as indicated and prelabeled with *myo*[^{3}H]inositol for 40 hr. Accumulation of [^{3}H]inositol phosphates under basal and stimulated conditions was determined as described. Abbreviations are as in Fig. 2.

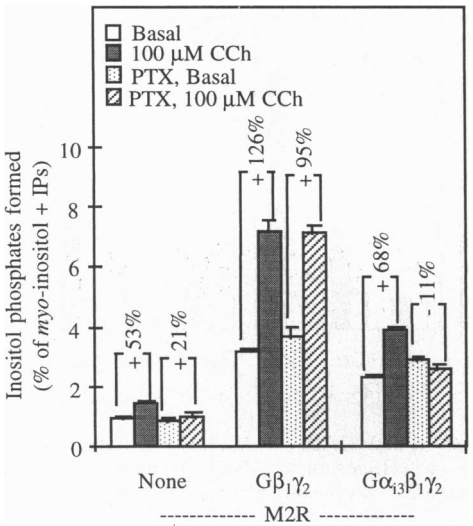

FIG. 4. Effect of PTX on PLC activity stimulated by M2R. COS-M6 cells were transfected with plasmids containing cDNAs as indicated and prelabeled with *myo*[^{3}H]inositol for 40 hr. Cells were treated with 0.1 μg of PTX per ml for 14 hr prior to the assay. Accumulation of [^{3}H]inositol phosphates under basal and stimulated conditions was determined as described.

the efficacy of the transduction process mediated by G_q varied significantly from receptor to receptor, making some G_s-coupled receptors more likely stimulators of PLC than others. For the transfected receptors the rank order of efficacy for G_q-mediated activation of PLC was V2R > LHR > β1AR > β2AR. The requirement for a high receptor expression level was evidenced by the fact that the endogenous COS cell β2AR (19) was unable to mediate stimulation of PLC (data not shown). Although not specifically studied, the different efficacy with which the different receptors stimulated PLC in cells coexpressing $G\alpha_q$ is likely to be the sum of the effect of differing affinities between G_q and the different receptors plus differences in the specific rates at which each receptor catalyzes the nucleotide exchange and subunit dissociation that gives $G\alpha_q$-GTP plus $G\beta\gamma$, the active PLC-stimulating forms of G_q.

Third, all the G_s-coupled receptors tested, as well as the G_i-coupled M2R, were capable of stimulating $G\beta\gamma$-stimulated PLC activity, indicating that full stimulation of PLC activity by G_s- and G_i-coupled receptors requires the participation of a $G\alpha$ subunit. Although this could have been mediated by G_{16} (13), this G protein does not appear to be the mediator of the phenomenon observed here, because it is not expressed in COS cells. Absence of $G\alpha_{16}$ in COS cells was shown by Simon and collaborators both by immunoblotting (20) and by the fact that PLC stimulation by interleukin 8 receptors is totally suppressed by PTX (21). Interleukin 8 receptors have the capacity to promote activation of PLC in COS cells through cotransfected G_{16}, but when they do the effect is insensitive to PTX (21).

Finally, our results uncovered the existence of a synergistic interaction between $G\beta\gamma$ dimers and an undefined receptor-activated G protein. This G protein, like G_{16}, appears to interact nonselectively with G_s- and G_i-coupled receptors, because all receptors were able to elicit a similar absolute increase in PLC activity. The synergy was well seen with β2AR and M2R, the receptors that were weakest in stimulating PLC activity on their own. Thus, in COS cells transfected with $G\beta\gamma$ plus β2AR or M2R, agonist-stimulated PLC activity was much more than the sum of the agonist-stimulated activity obtained in cells expressing receptor alone plus the activity obtained in cells expressing $G\beta\gamma$ and receptor but tested in the absence of agonist. This phenomenon, the synergistic stimulation of PLC activity resulting from interaction between $G\beta\gamma$ and a nonspecifically activated G protein, may contribute significantly to the widely observed dual signaling capacity of G_s- and G_i-coupled receptors by which they not only modulate the expected effector—i.e., adenylyl cyclase—but also PLCs.

Neither the identity of the G protein that synergizes with $G\beta\gamma$ in stimulating PLC activity, nor that of the PLC(s) that are stimulated, nor the mechanism by which synergy comes about are known at this time. But we can speculate about some of the possibilities. With regard to the identity of the receptor-activated G protein synergizing with $G\beta\gamma$, an analysis of the effects of carbachol in cells expressing the M2R and of isoproterenol in cells expressing the β2AR, allows us to rule out several candidates: (*i*) It is neither G_s nor G_i, for coexpression of the α subunits of these G proteins, rather than facilitating or leaving unaltered stimulation of $G\beta\gamma$-stimulated PLC activity by agonist (isoproterenol or carbachol), led to a reduction in activity, as expected if these $G\alpha$ subunits acted simply as scavengers. (*ii*) It is also neither another G_i nor a G_o, nor any of the other G proteins with an α subunit that is susceptible to ADP-ribosylation by PTX. In cells expressing M2R, the agonist-induced stimulation of the $G\beta\gamma$-stimulated PLC activity was PTX insensitive. (*iii*) It is also not G_q, for if this were the case one would have expected to see carbachol-stimulated PLC activities in COS cells expressing M2R and $G\alpha_q$ to be similar to those seen in COS cells expressing $G\beta\gamma$ and M2R. The carbachol-stimulated PLC activities in cells expressing M2R and $G\alpha_q$ were significantly lower than those seen in cells expressing M2R and $G\beta\gamma$. The same was seen when the effect of isoproterenol in cells expressing β2AR plus G_q was compared to that obtained in cells expressing β2AR plus $G\beta\gamma$.

Candidates among the known G protein α subunits synergizing with $G\beta\gamma$ in stimulating PLC are G_z, G_{11}, G_{14}, G_{12}, and G_{13}. $G\alpha_z$ is a structural homologue of $G\alpha_i$s that is likely to interact with M2R. But its capacity to activate PLCs has not yet been studied. $G\alpha_{11}$ and $G\alpha_{14}$ are structural homologues of G_q and have the potential of activating PLC. Neither $G\alpha_{12}$ nor $G\alpha_{13}$ is a substrate for PTX and for neither is a specific function yet known. It may be that the $G\beta\gamma$-stimulated PLC and that stimulated by receptor are the same and that the $G\beta\gamma$ and/or the receptor-activated G protein regulate PLC activity indirectly—i.e., secondarily to other changes in the cell metabolism such as activation of protein kinase C or increase in intracellular Ca^{2+} followed (or not) by activation of kinase(s) and/or phosphatase(s). In addition, two separate PLCs could be involved, one being $G\beta\gamma$ responsive and leading to activation of a kinase, the second being unresponsive to the (direct or indirect) action of the receptor-activated G protein, but becoming responsive to the receptor-generated signal due to the activity of $G\beta\gamma$. The finding that the absolute magnitude of the receptor-stimulated PLC activity in cells expressing $G\beta\gamma$ is about the same, regardless of receptor tested, is consistent with the possibility that the final response is due to two PLCs: one responsive to $G\beta\gamma$ (e.g., PLCβ2 or PLCβ3) and the other, as yet unidentified in molecular terms, becoming responsive to the signal generated by receptor in a manner that is conditional on direct or indirect costimulation by $G\beta\gamma$.

At the level of adenylyl cyclase, $G\alpha_s$ and $G\beta\gamma$ have been shown to potentiate each other's stimulatory effects by interacting at separate sites. This has led to the concept of conditional or coincidence regulation of cAMP formation, such that $G\beta\gamma$ is essentially ineffective unless there is coincident stimulation by $G\alpha_s$ (22). The data obtained here raise the possibility that a parallel situation may exist at the level of PLC whereby the intensity of the stimulatory effects of $G\beta\gamma$ dimers would be modulated by or even be dependent on costimulation by a $G\alpha$ subunit.

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