G Protein-coupled Receptor-induced Sensitization of Phospholipase C Stimulation by Receptor Tyrosine Kinases*  

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Martina Schmidt‡, Markus Frings, Marie-Luise Mono, Yuanjian Guo§, Paschal A. Oude Weernink, Sandrine Evellin, Li Han, and Karl H. Jakobs  
From the Institut für Pharmakologie, Universitätssklinikum Essen, D-45122 Essen, Germany  

Activation of stably expressed M₄ and M₅ muscarinic acetylcholine receptors (mAChRs) as well as of endogenously expressed lysophosphatidic acid and purinergic receptors in HEK-293 cells can induce a long lasting potentiation of phospholipase C (PLC) stimulation by these and other G protein-coupled receptors (GPCRs). Here, we report that GPCRs can induce an up-regulation of PLC stimulation by receptor tyrosine kinases (RTKs) as well and provide essential mechanistic characteristics of this sensitization process. Pretreatment of HEK-293 cells for 2 min with carbachol, a mAChR agonist, lysophosphatidic acid, or ATP, followed by agonist washout, strongly increased (by 2–3-fold) maximal PLC stimulation (measured >40 min later) by epidermal growth factor and platelet-derived growth factor, but not insulin, and largely enhanced PLC sensitivity to these RTK agonists. The up-regulation of RTK-induced PLC stimulation was cycloheximide-insensitive and was observed for up to ~90 min after removal of the GPCR agonist. Sensitization of receptor-induced PLC stimulation caused by prior M₄ mAChR activation was fully prevented by pertussis toxin and strongly reduced by expression of Gβγ scavengers. Furthermore, inhibition of conventional protein kinase C (PKC) isoenzymes and chelation of intracellular Ca²⁺ suppressed the sensitization process, while overexpression of PKC-α, but not PKC-β, further enhanced the M₄ mAChR-induced sensitization of PLC stimulation. None of these treatments affected acute PLC stimulation by either GPCR or RTK agonists. Taken together, short term activation of GPCRs can induce a strong and long lasting sensitization of PLC stimulation by RTKs, a process apparently involving G₁α-derived Gβγ as well as increases in intracellular Ca²⁺ and activation of a PKC isoenzyme, most likely PKC-α.

Stimulation of phosphoinositol-hydrolyzing phospholipase C (PLC) is a cellular response to activation of a large variety of membrane receptors, including numerous G protein-coupled receptors (GPCRs) as well as several receptor tyrosine kinases (RTKs). These two types of membrane receptors generally stimulate distinct PLC isoenzymes. GPCRs activate PLC-β isoenzymes, either via GTP-ligated α subunits of the G₁α class of G proteins or by βγ dimers liberated from G₁ type G proteins. In contrast, RTKs, such as those for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), activate PLC-γ isoenzymes by recruitment of these PLC enzymes to the autophosphorylated RTKs and subsequent tyrosine phosphorylation (1, 2). The hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC enzymes results in the generation of the two second messengers, inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol, which induce Ca²⁺ release from intracellular stores and activation of protein kinase C (PKC) isoforms, respectively. It is generally accepted that by these functional consequences stimulation of PLC enzymes plays a major role in many early and late cellular responses to receptor activation, such as smooth muscle contraction, secretion, neuronal signaling, and cell growth and differentiation, to name but a few (3–6). Thus, alteration in receptor signaling to PLC enzymes is expected to have a major impact on cellular responses evoked by this receptor.

We reported recently that short term activation of GPCRs in HEK-293 cells stably expressing the M₄ or M₅ subtypes of muscarinic acetylcholine receptors (mAChRs) can induce a long lasting potentiation of PLC stimulation by these and other GPCRs, including the endogenously expressed lysophosphatidic acid (LPA) and purinergic receptors (7–9). Studies with pertussis toxin (PTX) and PKC inhibitors, furthermore, suggested that this potentiation of PLC stimulation by GPCRs is mediated by G₁ type G proteins and involves activation of a PKC isoenzyme (8, 9). Since GPCRs and RTKs activate distinct PLC isoenzymes and by distinct mechanisms, we wondered whether GPCRs may also induce sensitization of PLC stimulation by RTKs endogenously expressed in HEK-293 cells (10, 11). We report here that short term activation of GPCRs can induce a long lasting up-regulation of PLC stimulation by EGF and PDGF but not insulin. Furthermore, evidence is provided suggesting that this sensitization of PLC stimulation is mediated by G₁α-derived Gβγ dimers and that increases in cytosolic Ca²⁺ and activation of a conventional PKC enzyme, most likely PKC-α, are required for this novel PLC regulatory mechanism.

EXPERIMENTAL PROCEDURES

Materials—myo-[³H]Insitol (10–25 Ci/mol), n-myo-[³H]InsP₃ (21 Ci/mol), and N-[³H]methylsulfolamine ([³H]NMS; 84 Ci/mol) were
tylocholine receptor; LPA, lysophosphatidic acid; PTX, pertussis toxin; NMS, N-methylsulfolamine; β-ARK-CT, carboxy terminus of the β-adrenergic receptor kinase; BAPTA/AM, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid acetyl methyl ester.

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‡ To whom correspondence should be addressed: Institut für Pharmakologie, Universitätssklinikum Essen, Hufelandstrasse 55, D-45122 Essen, Germany. Tel.: 49-201-723-3457; Fax: 49-201-723-5968; E-mail: martina.schmidt@uni-essen.de.
§ Present address: Dept. of Molecular Pharmacology, SUNY, Stony Brook, NY 11794-8651.

The abbreviations used are: PLC, phospholipase C; GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; InsP₃, inositol 1,4,5-trisphosphate; PKC, protein kinase C; mAChR, muscarinic acetylcholine receptors; GPCRs, G protein-coupled receptors; RTKs, receptor tyrosine kinases; PDGF, platelet-derived growth factor; NMS, N-methylsulfolamine; β-ARK-CT, carboxy terminus of the β-adrenergic receptor kinase; BAPTA/AM, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid acetyl methyl ester.

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from NEN Life Science Products. Unlabeled d-myO-InsP$_3$, PDGF-BB, and EGF were from Biomol; insulin (1-2767; human recombinant expressed in Escherichia coli, sodium salt, crystalline), LPA, and cycloheximide were from Sigma; and Go 6976 and BAPTA/AM were from Calbiochem. The antibodies, MC5, which recognizes PkC-α, -β, and -γ isoforms, and C-20, which recognizes all Go$_s$, isoforms including Go$_s$ from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other materials were from previously described sources (7–9).

**Cell Culture and Transfection**—DNAs encoding PKC-α and PKC-βI both subcloned into pRKS were kindly provided by Drs. M. Kellerer and H. Mischak. DNA encoding the carboxyl terminus of the β-adrenergic receptor and C-20, which recognizes all Go$_s$, isoforms including Go$_s$ from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other materials were from previously described sources (7–9).

**Agonist Pretreatment and Measurement of Inositol Phosphate Formation**—Cellular phospholipids were labeled by incubating cells for 36 h with myo-[3H]inositol (0.5 μCi/ml in serum-free medium. Where indicated, cells were incubated during the last 16 h of the labeling period with 100 ng/ml PTX. Afterward, the labeling medium was removed, and the adherent cells were equilibrated for 10 min at 37°C in Hanks’ balanced salt solution, containing 118 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 5 mM glucose, buffered at pH 7.4 with 15 mM HEPES. Thereafter, the cells were incubated for 2 min at 37°C in Hanks’ balanced salt solution with and without the indicated receptor agonist in the absence of LiCl, followed by thorough washout of the agonist and further incubation of the cells for 30 min or the indicated periods of time without agonist as reported before (8, 9). Then the adherent cells were incubated for 10 min at 37°C with 10 mM LiCl in Hanks’ balanced salt solution, immediately followed by the addition of stimulatory agents in the presence of 10 mM LiCl to measure the formation of total [3H]inositol phosphates (usually for 30 min at 37°C) as described before (16). To study the effects of cycloheximide, Go 6976, and BAPTA/AM on PLC stimulation, the cells were pretreated for 60 min (cycloheximide) or 30 min (Go 6976, BAPTA/AM) with these agents or their solvent, dimethyl sulfoxide (0.1 or 0.2%). These agents were also present during agonist pretreatment, subsequent incubation without agonist, and final PLC assays.

**InsP$_3$ Mass Determination**—Unlabeled HEK-293 cells serum-starved for 36 h were treated for 2 min with and without receptor agonist, followed by agonist washout and, 30 min later, treatment for 10 min with 10 mM LiCl as described above. Then the adherent cells were incubated for 15 s at 37°C with and without EGF or PDGF. InsP$_3$ mass was determined by a radioreceptor assay as described before (9, 17, 18). In rapid and concentration-dependent accumulation of [3H]inositol phosphates. As illustrated in Fig. 1 for M$_2$ mAChR-expressing HEK-293 cells, at maximally effective concentrations, EGF (50 ng/ml), PDGF (20 ng/ml), and insulin (10 μg/ml) increased [3H]inositol phosphate production determined 30 min after agonist addition by 2–3-fold above basal level. The formation of [3H]inositol phosphates induced by the three RTK agonists was rather linear with time for up to 30 min of incubation. PLC stimulation by EGF and PDGF, which was also monitored as rapid InsP$_3$ accumulation (see Fig. 3), was specifically inhibited by the EGF receptor-specific tyrphostin AG 1478 (1 μM) and the PDGF receptor-specific tyrphostin AG 1296 (10 μM) (20, 21), respectively, without altering PLC stimulation by other RTK agonists (data not shown). Treatment of the cells with PTX (100 ng/ml, 16 h) did not affect PLC stimulation by any of the three RTK agonists (data not shown; see Fig. 8). GPCR-induced Sensitization of PLC Stimulation by RTKs—To study whether GPCRs can induce sensitization of PLC stimulation by RTKs, M$_2$ mAChR-expressing HEK-293 cells were first treated for 2 min with the mAChR agonist, carbachol (1 mM), followed by agonist washout, a further 40-min incubation without any agonist, and then measurement of basal and agonist-stimulated accumulation of [3H]inositol phosphates. As reported before (9), basal [3H]inositol phosphate accumulation was not altered in carbachol-pretreated compared with control cells, while [3H]inositol phosphate formation induced by re-stimulation of the cells with 1 mM carbachol was significantly increased by about 60% (n = 4, p < 0.01). As illustrated in Fig. 2, prestimulation of the cells with carbachol also markedly enhanced PLC stimulation by EGF and PDGF. At 40 min after the 2-min treatment with carbachol, [3H]inositol phosphate production induced by EGF (50 ng/ml) was enhanced 2-fold, from 2.95 ± 0.45 to 5.9 ± 0.51 × 10$^3$ cpm/mg of protein (n = 4, p < 0.01) (Fig. 2A). Similarly, [3H]inositol phosphate formation induced by PDGF (20 ng/ml) was increased from 3.05 ± 0.23 × 10$^3$ cpm/mg of protein in untreated control cells to 5.85 ± 0.25 × 10$^3$ cpm/mg of protein in carbachol-pretreated cells (n = 4, p < 0.01) (Fig. 2B). Under the same conditions, PLC stimulation by insulin was not altered in carbachol-pretreated compared with control cells (Fig. 2C). The up-regulation of agonist-induced [3H]inositol phosphate formation was fully blocked by the mAChR antagonist, atropine (10 μM), added during pre-treatment of the cells with 1 mM carbachol (data not shown). Similar to the enhancement of EGF- and PDGF-stimulated [3H]inositol phosphate formation, pretreatment of the cells with carbachol also markedly increased rapid formation of InsP$_3$ by the RTK agonists. As shown in Fig. 3, stimulation of
control cells for 15 s with 50 ng/ml EGF or 20 ng/ml PDGF increased InsP₃ levels about 2-fold. At 40 min after carbachol treatment of the cells, this RTK agonist-induced increase in InsP₃ levels was enlarged by 2–3-fold.

Under the conditions studied, the M₂ mAChR-induced up-regulation of PLC stimulation by EGF and PDGF was maximal at 40 min after carbachol removal, the earliest time point examined, and declined thereafter (Fig. 4). Even at 85 min after carbachol removal, EGF- and PDGF-induced [³H]inositol phosphate formation was significantly (p < 0.05) enhanced compared with untreated control cells, while at 145 min, control responses were again obtained. Thus, short term M₂ mAChR activation of HEK-293 cells caused a long lasting up-regulation of PLC stimulation by the RTK agonists, EGF and PDGF. The up-regulation of PLC stimulation induced by carbachol pretreatment was apparently not dependent on the synthesis of a protein causing this long lasting effect. As studied for EGF-stimulated PLC activity, pretreatment of HEK-293 cells for 1 h with 350 μM cycloheximide decreased [³H]inositol phosphate formation in control cells from 2.58 ± 0.16 to 1.83 ± 0.05 x 10³ cpm/mg of protein (n = 3). However, the up-regulation of EGF-stimulated PLC activity induced by a 2-min pretreatment of the cells with 1 mM carbachol was not altered by prior cycloheximide treatment. In cells pretreated with carbachol, EGF increased [³H]inositol phosphate formation in control and cycloheximide-pretreated cells by 4.13 ± 0.07 and 3.38 ± 0.08 x 10³ cpm/mg of protein, respectively (n = 3) (data not shown).

EGF- and PDGF-Induced PLC stimulation in M₂ mAChR-expressing HEK-293 cells was also up-regulated by activation of the endogenously expressed LPA receptor. Similar to carbachol, pretreatment of the cells for 2 min with 10 μM LPA, at 40 min after carbachol removal, the earliest time point examined, and declined thereafter (Fig. 4). Even at 85 min after carbachol removal, EGF- and PDGF-induced [³H]inositol phosphate formation was significantly (p < 0.05) enhanced compared with untreated control cells, while at 145 min, control responses were again obtained. Thus, short term M₂ mAChR activation of HEK-293 cells caused a long lasting up-regulation of PLC stimulation by the RTK agonists, EGF and PDGF. The up-regulation of PLC stimulation induced by carbachol pretreatment was apparently not dependent on the synthesis of a protein causing this long lasting effect. As studied for EGF-stimulated PLC activity, pretreatment of HEK-293 cells for 1 h with 350 μM cycloheximide decreased [³H]inositol phosphate formation in control cells from 2.58 ± 0.16 to 1.83 ± 0.05 x 10³ cpm/mg of protein (n = 3). However, the up-regulation of EGF-stimulated PLC activity induced by a 2-min pretreatment of the cells with 1 mM carbachol was not altered by prior cycloheximide treatment. In cells pretreated with carbachol, EGF increased [³H]inositol phosphate formation in control and cycloheximide-pretreated cells by 4.13 ± 0.07 and 3.38 ± 0.08 x 10³ cpm/mg of protein, respectively (n = 3) (data not shown).

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followed by agonist washout and measurement of \( ^{3}H \)inositol phosphate formation 40 min later, increased PLC stimulation by EGF and PDGF, without altering basal \( ^{3}H \)inositol phosphate accumulation. Maximal EGF-induced \( ^{3}H \)inositol phosphate formation was increased by 55\% (\( n = 5, p < 0.01 \)) (Fig. 5A), and that induced by PDGF was increased by 62\% (\( n = 3, p < 0.01 \)) (Fig. 5B). The up-regulation of PLC stimulation by EGF and PDGF caused by pretreatment of the cells with LPA was even more evident at low concentrations of the RTK agonists. For example, 7.5 ng/ml PDGF only slightly increased \( ^{3}H \)inositol phosphate formation in control cells, whereas in LPA-pretreated cells PDGF at the same concentration caused a strong increase in \( ^{3}H \)inositol phosphate formation, reaching the same level as the maximal PDGF-induced stimulation in control cells (Fig. 5B).

Sensitization of RTK-induced PLC stimulation caused by short term activation of GPCRs was also observed in wild-type cells and HEK-293 cells overexpressing the M<sub>3</sub> mAChR. As shown in Fig. 6, treatment of either cell type for 2 min with 10 \( \mu \)M LPA, followed by washout of LPA and measurement of \( ^{3}H \)inositol phosphate formation 40 min later, strongly increased EGF-induced PLC stimulation. In wild-type cells, maximal EGF-induced \( ^{3}H \)inositol phosphate formation was increased by 157\% (\( n = 3, p < 0.01 \)) (Fig. 6A), and that induced by EGF in M<sub>3</sub> mAChR-expressing cells was increased by prior treatment with LPA by 134\% (\( n = 4, p < 0.01 \)) (Fig. 6B). Furthermore, pretreatment of M<sub>3</sub> mAChR-expressing HEK-293 cells for 2 min with 1 mM carbachol increased PLC stimulation by EGF (50 ng/ml) measured 40 min later by 90\% (\( n = 3, p < 0.01 \)) (data not shown). Finally, as demonstrated in Fig. 7, short term (2 min) activation of the endogenously expressed purinergic receptor with ATP (1 mM), followed by washout of ATP and measurement of \( ^{3}H \)inositol phosphate formation 70 min later, not only enhanced PLC stimulation by carbachol (1 mM) in M<sub>2</sub> mAChR-expressing cells (by 60\%, \( n = 4, p < 0.01 \)) as reported before (9) but also largely increased EGF-stimulated PLC activity in these as well as in wild-type HEK-293 cells. Maximal EGF-induced \( ^{3}H \)inositol phosphate formation was increased in M<sub>2</sub> mAChR-expressing cells by 88\% (\( n = 4, p < 0.01 \)) (Fig. 7A), and that induced by EGF in wild-type cells was increased by prior treatment with ATP by 182\% (\( n = 3, p < 0.01 \)) (Fig. 7B). Thus, short term activation of various endogenously expressed or overexpressed GPCRs in HEK-293 cells strongly increased maximal PLC stimulation by EGF and PDGF as well as the sensitivity to the RTK agonists.

Since prior GPCR activation increased subsequent PLC stimulation by both RTKs (for EGF and PDGF) and GPCRs (7–9), we examined in M<sub>2</sub> mAChR-expressing HEK-293 cells whether cell surface receptor number is altered by this treatment. For this, the cells were treated for 2 min without and with 1 mM carbachol, 10 \( \mu \)M LPA, or 1 mM ATP, followed by washout of the agonists and measurement of M<sub>2</sub> mAChR number 40 min later (carbachol, LPA) or 70 min later (ATP), thus exactly under the conditions in which the M<sub>2</sub> mAChR-induced

![Fig. 5. LPA-induced sensitization of EGF- and PDGF-stimulated inositol phosphate formation. M<sub>3</sub> mAChR-expressing HEK-293 cells prelabeled with \( ^{3}H \)inositol were pretreated for 2 min without (Control) and with 10 \( \mu \)M LPA (LPA-pretreated). At 40 min after LPA washout, the formation of \( ^{3}H \)inositol phosphates was determined at the indicated concentrations of EGF (A) or PDGF (B). Similar data were obtained in 3–5 independent experiments.](image5.png)

![Fig. 6. LPA-induced sensitization of EGF-stimulated PLC activity in wild-type and M<sub>3</sub> mAChR-expressing HEK-293 cells. Wild-type (A) and M<sub>3</sub> mAChR-expressing (B) HEK-293 cells prelabeled with \( ^{3}H \)inositol were pretreated for 2 min without (Control) and with 10 \( \mu \)M LPA (LPA-pretreated). At 40 min after LPA washout, the formation of \( ^{3}H \)inositol phosphates was determined at the indicated concentrations of EGF. Similar data were obtained in three or four independent experiments.](image6.png)
The number of cell surface M2 mAChRs was determined in three or four independent experiments. Pretreatment with the PTX-sensitive up-regulation of PLC responses was examined. The up-regulation of PLC stimulation caused by prior carbachol treatment was fully prevented. Since the M2 mAChR inhibits adenyl cyclase via PTX-sensitive G proteins and decreases cAMP levels in these cells (13), we examined whether the M2 mAChR-induced up-regulation of PLC stimulation may be caused by a fall in cAMP levels. However, treatment of the cells for 30 min with the membrane-permeable cAMP analog, dibutyryl cAMP (1 mM), neither altered PLC stimulation by carbachol and EGF in control cells nor affected the up-regulation of PLC stimulation caused by prior carbachol treatment (data not shown).

Receptor-activated G proteins transmit the signal to effectors either by the GTP-ligated α subunits or by the released free Gβγ dimers (22, 23). To study whether Gβγ dimers mediate the PTX-sensitive up-regulation of PLC responses, we examined the effects of expression of the two Gβγ scavengers, β-ARK-CT and Gαq (12), on acute PLC stimulation and its sensitization caused by pretreatment of the cells with carbachol. Expression of β-ARK-CT or Gαq did not alter basal PLC activity (data not shown) and PLC stimulation by carbachol and EGF in control untreated cells (Fig. 9). In contrast, the up-regulation of PLC stimulation by carbachol and EGF induced by pretreatment of the cells with carbachol was largely reduced by expression of β-ARK-CT or Gαq. In β-ARK-CT-expressing HEK-293 cells, carbachol increased [3H]inositol phosphate formation in control and carbachol-pretreated cells by 4.32 ± 0.49 and 4.55 ± 0.20 x 10^3 cpm/mg of protein (n = 4), respectively, and that induced by EGF amounted to 2.82 ± 0.23 and 3.37 ± 0.47 x 10^3 cpm/mg of protein (n = 4), respectively (Fig. 9A). In Gαq-expressing cells, [3H]inositol phosphate formation was increased by carbachol in control and carbachol-pretreated cells by 6.00 ± 0.50 and 6.37 ± 0.42 x 10^3 cpm/mg of protein (n = 3), respectively, and that induced by EGF amounted to 2.51 ± 0.51 and 2.76 ± 0.32 x 10^3 cpm/mg of protein (n = 3), respectively (Fig. 9B).

Role of PKC and Ca^{2+} in M2 mAChR-induced Sensitization of PLC Stimulation—Pretreatment of HEK-293 cells for 30 min with the PKC inhibitor, Gö 6976 (100 nM), did not affect PLC stimulation by either carbachol or EGF in control cells. However, the up-regulation of PLC stimulation by carbachol and EGF caused by prior carbachol treatment was completely prevented. Since the M2 mAChR inhibits adenyl cyclase via PTX-sensitive G proteins and decreases cAMP levels in these cells (13), we examined whether the M2 mAChR-induced up-regulation of PLC stimulation may be caused by a fall in cAMP levels. However, treatment of the cells for 30 min with the membrane-permeable cAMP analog, dibutyryl cAMP (1 mM), neither altered PLC stimulation by carbachol and EGF in control cells nor affected the up-regulation of PLC stimulation caused by prior carbachol treatment (data not shown).

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mAChR-induced sensitization of PLC stimulation, we examined the effects of overexpression of PKC-α and PKC-βI on PLC stimulation. Overexpression of either PKC isoenzyme had no effect on PLC stimulation by carbachol or EGF in naive cells (Fig. 11). However, in cells overexpressing PKC-α, the increase in carbachol- and EGF-induced PLC stimulation caused by pretreatment of the cells with carbachol was strongly enhanced (Fig. 11A). In carbachol-pretreated cells, rechallenge with carbachol increased [3H]inositol phosphate formation by 12.9 ± 0.78 × 10³ cpm/mg of protein in control cells and by 16.2 ± 9.3 × 10³ cpm/mg of protein in cells overexpressing PKC-α (n = 4, p < 0.01). Similarly, in carbachol-pretreated cells, EGF-induced [3H]inositol phosphate formation was enhanced from 4.85 ± 0.62 × 10³ cpm/mg of protein in control cells to 7.52 ± 0.49 × 10³ cpm/mg of protein in cells overexpressing PKC-α (n = 4, p < 0.01). This enhancement of M₄ mAChR-induced sensitization of PLC stimulation was fully blocked by G6 6976 (data not shown). In contrast to PKC-α, overexpression of PKC-βI did not alter the M₄ mAChR-induced sensitization of PLC stimulation (Fig. 11B).

Since the GPCRs that induced sensitization of PLC stimulation also markedly increase cytosolic Ca²⁺ concentration in HEK-293 cells (16, 25), we finally examined whether this increase is involved in sensitization of PLC stimulation. For this, the cells were treated before carbachol treatment with the intracellular Ca²⁺ chelator, BAPTA/AM (20 μM, 30 min), which completely prevented the agonist-induced increase in cytosolic [3H]inositol phosphate formation stimulated by 1 mM carbachol or 50 ng/ml EGF was determined. Data are representative of four independent experiments.

**FIG. 9.** Inhibition of M₄ mAChR-induced PLC sensitization by Gβγ scavengers. M₄ mAChR-expressing HEK-293 cells were transfected with empty vectors (−), β-ARK-CT (+ in A), or Gα₁ (+ in B) (100 μg of DNA each) and labeled with myo-[3H]inositol. At 48 h after transfection, the cells were treated for 2 min without (Control) and with 1 mM carbachol (Carbachol-pretreated). At 40 min after carbachol washout, [3H]inositol phosphate formation stimulated by 1 mM carbachol or 50 ng/ml EGF was determined. Data are representative of three or four independent experiments. *Inset,* immunoblot detection of Gα₁ in lysates of transfected cells. The band seen in vector-transfected cells (Ctr) and migrating above Gα₁ represents Gα proteins recognized by the antibody (C-20) used.

**FIG. 10.** Inhibition of M₄ mAChR-induced PLC sensitization by Gαi scavengers. M₄ mAChR-expressing HEK-293 cells were transfected with empty vectors (−), or Gα₈ (± in B) (100 μg of DNA each) and labeled with myo-[3H]inositol. At 48 h after transfection, the cells were treated for 2 min without (Control) and with (+) 100 nM G6 6976. Then the cells were treated for 2 min without (Control) and with 1 mM carbachol (Carbachol-pretreated). At 40 min after carbachol washout, [3H]inositol phosphate (InsP₃) formation stimulated by 1 mM carbachol or 50 ng/ml EGF was determined. Data are representative of four independent experiments.

**FIG. 11.** Enhancement of M₄ mAChR-induced PLC sensitization by PKC-α. M₄ mAChR-expressing HEK-293 cells were transfected with empty vector (−), PKC-α (+ in A), or PKC-βI (+ in B) (25 μg of DNA each) and labeled with myo-[3H]inositol. At 48 h after transfection, the cells were treated for 2 min without (Control) and with 1 mM carbachol (Carbachol-pretreated). At 40 min after carbachol washout, [3H]inositol phosphate formation stimulated by 1 mM carbachol or 50 ng/ml EGF was determined. Data are representative of four independent experiments. *Inset,* immunoblot detection of PKC-α and PKC-βI in lysates of transfected cells.
Sensitization of PLC Signaling by RTKs

Fig. 12. Inhibition of M2 mAChR-induced PLC sensitization by BAPTA/AM. M2 mAChR-expressing HEK-293 cells prelabeled with myo-[^3H]inositol were first treated for 30 min without (−) and with (+) 20 μM BAPTA/AM. Then the cells were treated for 2 min without (Control) and with 1 mM carbachol (Carbachol-pretreated). At 40 min after carbachol washout, [^3H]inositol phosphate (InsP) formation stimulated by 1 mM carbachol or 50 ng/ml EGF was determined. Data are representative of five independent experiments.

Ca^2+ concentration (data not shown). As shown in Fig. 12, in cells pretreated with BAPTA/AM, PLC stimulation caused by carbachol or EGF in control cells was not altered. However, the BAPTA/AM treatment completely abolished the carbachol-induced up-regulation of PLC stimulation caused by either carbachol or EGF.

**DISCUSSION**

We reported before that short term activation of GPCRs in HEK-293 cells stably expressing the M2 or M3 mAChR subtypes can induce a long lasting sensitization of PLC stimulation by these and other GPCRs. The GPCR-induced up-regulation of PLC stimulation was prevented by PTX and inhibition of PKC enzymes (7–9). Since GPCRs and RTKs activate distinct PLC isoenzymes, GPCRs mainly PLC-β enzymes and RTKs PLC-γ enzymes, particularly the widely expressed PLC-γ1 (1, 2), a major aim of the present report was to examine whether GPCRs can induce potentiation of PLC stimulation by RTKs as well. Furthermore, the mechanisms involved in this up-regulation were explored, particularly whether up-regulation of PLC stimulation by GPCRs and RTKs involves identical or distinct mechanisms. For the study, we used wild-type HEK-293 cells as well as HEK-293 cells stably expressing the M2 or M3 mAChR subtypes and endogenously expressing various other GPCRs as well as RTKs for EGF, PDGF, and insulin (8–11). We report here that short term activation of the overexpressed M2 and M3 mAChRs and the endogenously expressed LPA and purinergic receptors can induce a strong and long lasting up-regulation and sensitization of PLC stimulation by EGF and PDGF but not insulin. Furthermore, evidence is provided that the GPCR-induced sensitization of PLC stimulation is apparently mediated by Gβγ dimers liberated from PTX-sensitive G1 type G proteins and requires increases in cytosolic Ca^2+ concentration and activation of a conventional PKC enzyme, most likely PKC-α. Finally, extensive comparison of PLC stimulation by the M2 mAChR and the EGF receptor strongly suggests that very similar or even identical mechanisms are involved in the process of sensitization of PLC stimulation by these two distinct receptor types.

The enhancement of PLC stimulation by EGF and PDGF induced by prior GPCR activation was apparently not due to a block or inhibition of a desensitization process. First, accumulation of inositol phosphates induced by EGF and PDGF in control cells was rather linear with time for up to 30 min; thus, there was no major desensitization of PLC stimulation during this time period. Second, similar to the enhancement of inositol phosphate accumulation measured over a 30-min period, prior GPCR treatment of HEK-293 cells also strongly increased EGF- and PDGF-stimulated formation of InsP3, measured 15 s after challenge of the cells with the RTK agonists. Interestingly, PLC stimulation in HEK-293 cells by insulin was not increased by prior activation of the M2 mAChR. The reason for this discrepancy is presently not clear. While stimulation of PLC activity, particularly of the PLC-γ1 enzyme, is a well established and rather general response to EGF and PDGF receptor activation, it is not so for insulin (1, 2, 26, 27), although stimulation of PLC activity by insulin has been described in some cell types, and PLC-γ has recently been reported to participate in metabolic signaling by the insulin receptor in adipocytes (28–30). Regardless of the underlying mechanisms, the insensitivity of PLC stimulation by insulin to prior GPCR activation indicates that the sensitization of PLC stimulation by EGF and PDGF is not an unspecific response to any PLC stimulatory receptor.

The experimental paradigm used in the present study to demonstrate GPCR-induced sensitization of PLC stimulation by EGF and PDGF (i.e., first treatment of HEK-293 cells for a short period (2 min) with a GPCR agonist and then washout of this agonist and subsequent incubation of the cells for 40 min or longer without any agonist before actual measurement of PLC activity) is based on previous data on up-regulation of PLC stimulation by GPCRs. These studies on M2 and M3 mAChR-induced sensitization of PLC stimulation demonstrated that maximal up-regulation of PLC stimulation by these mACHRs is observed at about 40 min after washout of the initial stimulus and slowly disappears thereafter (8, 9). Using these experimental conditions, it is demonstrated that sensitization of PLC stimulation by EGF and PDGF caused by prior M2 mAChR activation is also a long lasting process, with a maximum at 40 min after the initial treatment with carbachol and a slow decline thereafter, reaching control values at ~150 min.

Not only the time courses but also the mechanisms involved in the GPCR-induced sensitization of PLC stimulation by GPCRs and RTKs are apparently very similar. Specifically, it is shown that the M2 mAChR-induced up-regulation of PLC stimulation by either carbachol or EGF is completely abrogated by PTX treatment of the cells and largely reduced by expression of the two Gβγ scavengers, βARK-CT and Goα. In addition, inhibition of conventional PKC enzymes with Go6976 and BAPTA/AM fully blocked the M2 mAChR-induced up-regulation of PLC stimulation by either carbachol or EGF. None of these treatments had an effect on PLC stimulation by carbachol or EGF in naive cells. Furthermore, as demonstrated for the M2 mAChR, the sensitization of PLC stimulation caused by prior GPCR activation was apparently not due to a corresponding up-regulation of cell surface receptor number. Thus, the sensitizing GPCRs apparently generate two distinct signals mediating the long lasting sensitization process of PLC stimulation. One signal is apparently caused by activation of PLC, which is PTX-insensitive in HEK-293 cells, thus most likely mediated by Gbg type G proteins, and finally results in Ca^2+ mobilization and PKC activation. The results obtained with Go6976 and BAPTA/AM prompted us to investigate which of the conventional Ca^2+-dependent PKC isoforms known to be inhibited by Go6976, PKC-α and PKC-βII (24), mediates the up-regulation of PLC stimulation. It

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*S. Evellin, L. Han, and K. H. Jakobs, unpublished data.*

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S. Schmidt, M. Frings, M.-L. Mono, Y. Guo, P. A. Oude Weernink, S. Evellin, L. Han, and K. H. Jakobs, unpublished data.
is shown that overexpression of PKC-α, which had no effect on PLC stimulation in naive cells, largely enhanced the M2 mAChR-induced sensitization of PLC stimulation by carbachol and EGF, whereas overexpression of PKC-β2 was without any effect. Similar negative results were obtained in cells overexpressing PKC-βII, PKC-ε, or PKC-ζ (data not shown). Thus, one major signal involved in and mediating the GPCR-induced sensitization of PLC stimulation is apparently the activation of a conventional Ca2+-dependent PKC isoenzyme, most likely PKC-α. It remains to be studied whether Ca2+ acts solely by activating the PKC enzyme or whether additional Ca2+-dependent steps are involved in the PLC sensitization process.

The second signal generated by the sensitizing GPCR is apparently dependent on Gβγ dimers derived from receptor-activated Gi type G proteins (31). During the last few years, various direct and indirect effectors of Gβγ dimers have been identified (22, 23). Since the M2 mAChR-induced PLC stimulation in naive cells was affected neither by PTX nor by expression of Gβγ scavengers, it is highly unlikely that a PLC-β isoenzyme known to be controlled by Gβγs (1, 2) is the relevant Gβγ effector. Thus, overall, the GPCR-induced sensitization of PLC stimulation by GPCRs and RTKs apparently requires the activation of a PLC-derived signal (i.e. increase in intracellular Ca2+ concentration and activation of a conventional PKC isoenzyme) and an as yet unidentified Gβγ effector, which then in combination induce a long lasting cellular memory for receptor-mediated PLC stimulation.

During the last years, various GPCRs have been reported to cause “transactivation” of RTKs, specifically of the EGF and PDGF receptors, in different cellular systems (for a review, see Ref. 32). These studies demonstrated that tyrosine phosphorylation of the EGF or PDGF receptor is an essential intermediate step particularly for mitogenic signaling by these GPCRs. The results presented in this report demonstrating GPCR-induced sensitization of PLC stimulation by EGF and PDGF receptors may also be termed “transactivation,” however with a completely different meaning. First, the experimental paradigm used to demonstrate GPCR-induced sensitization of PLC stimulation by RTKs is quite distinct from that used in the above mentioned “transactivation” studies, in which acute GPCR-induced cellular responses were shown to involve activation of a RTK. Second, in contrast to the GPCR-induced “transactivation” of EGF or PDGF receptors, which was independent of exogenous RTK agonists (32), the GPCR-induced PLC sensitization was only observed upon the addition of exogenous RTK (or GPCR) ligands, whereas agonist-independent basal PLC activity was not altered in GPCR-pretreated cells.

In conclusion, the data presented in this report demonstrate that short term activation of various GPCRs expressed in HEK-293 cells can induce a strong and long lasting up-regulation and sensitization of PLC stimulation by EGF and PDGF receptors, two prototypical RTKs. This novel cellular response apparently involves the complex interplay of at least two distinct signaling pathways induced by the GPCRs. The up-regulation and sensitization of PLC stimulation by RTKs described herein most likely has a major impact on physiological and possibly also pathological cellular responses triggered by these growth factor receptors.

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