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G_i- and Protein Kinase C-Mediated Heterologous Potentiation of Phospholipase C Signaling by G Protein-Coupled Receptors

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ABSTRACT

We recently reported that activation of the highly efficient phospholipase C (PLC) stimulatory m3 muscarinic acetylcholine receptor (mAChR) can induce a long-lasting G_i-mediated heterologous potentiation of PLC stimulation in human embryonic kidney (HEK) 293 cells, which was accompanied by an increased cellular level of the PLC substrate phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2]. Here, we examined whether such a potentiated PLC response is also induced by the rather poorly PLC stimulatory m2 mAChR and the endogenously expressed purinergic and lysophosphatidic acid receptors. Pretreatment of m2 mAChR-expressing HEK 293 cells for 2 min with carbachol, followed by agonist washout and measurement of PLC activity ≥40 min later, caused a long-lasting (up to ~90 min) heterologous potentiation of receptor- and G protein-mediated PLC stimulation. A similar heterologous potentiation of receptor-mediated PLC stimulation was induced by short term activation of lysophosphatidic acid and purinergic receptors. Either of the three receptor agonists increased the cellular level of PtdIns(4,5)P_2 by ~50%. The mAChR-induced PLC potentiation was fully prevented by either pertussis toxin or the protein kinase C (PKC) inhibitors staurosporine and Gö 6976, which did not affect acute PLC stimulation. On the other hand, the rise in PtdIns(4,5)P_2 was prevented only by combined treatment of HEK 293 cells with pertussis toxin and PKC inhibitors. In conclusion, we demonstrated that activation of poorly PLC stimulatory receptors can also induce a long-lasting G_i-mediated heterologous potentiation of PLC signaling in HEK 293 cells and that this novel PLC regulatory process is under the control of PKC.

Stimulation of PLC enzymes is an early cellular response of eukaryotic cells downstream of many tyrosine kinase receptors and receptors coupled to heterotrimeric G proteins. By hydrolyzing the minor membrane phospholipid PtdIns(4,5)P_2, PLC catalyzes the formation of the two second messenger molecules, Ins(1,4,5)P_3 and diacylglycerol, leading to increase in intracellular Ca^{2+} concentration and activation of PKC enzymes, respectively (Berridge, 1993; Nishizuka, 1995). Activation of G protein-coupled receptors by the appropriate agonists stimulates PLC isozymes of the PLC-β subtype by two distinct mechanisms. The PTX-insensitive activation of PLC-β enzymes is mediated by the α subunits of the G_q class of G proteins, whereas the PTX-sensitive stimulation of PLC-β isoforms seems to be caused by free $\beta\gamma$ dimers of G_i-type G proteins (Rhee and Bae, 1997). Similar to other transmembrane signaling processes, receptor signaling via PLC can exhibit rapid desensitization, as demonstrated for various receptors and cellular systems (Wojcikiewicz *et al*., 1993; Fischer, 1995).

Recently, we reported the rather unexpected finding that short term agonist pretreatment of HEK 293 cells stably expressing the human m3 mAChR subtype can cause a long-lasting heterologous potentiation of receptor- and G protein-mediated inositol phosphate formation (Schmidt *et al*., 1995a; Schmidt *et al*., 1996). Although acute PLC stimulation by m3 mAChRs is mediated by PTX-insensitive G_q-type G proteins (Hulme *et al*., 1990; Offermanns *et al*., 1994), potentiation of PLC responses was completely PTX sensitive, suggesting participation of G_i proteins. The potentiated PLC response was accompanied by a rise in cellular PtdIns(4,5)P_2 level (Schmidt *et al*., 1996). Compared with the m3 mAChR, the m2 mAChR preferentially couples to G_i-type G proteins, leading to, for example, inhibition of adenylyl cyclase (Peralta *et al*., 1988; Hulme *et al*., 1990). At high agonist concentrations, the m2 mAChR also stimulates PLC in HEK 293 cells. This stimulation, however, is PTX insensitive and, compared with m3 mAChR stimulation, rather poor (Peralta *et*

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ABBREVIATIONS: GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; LPA, lysophosphatidic acid; mAChR, muscarinic acetylcholine receptor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol-4-monophosphate; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PTX, pertussis toxin; HEK, human embryonic kidney.

al., 1988; Offermanns *et al*., 1994; Schmidt *et al*., 1995b). In the current study, we demonstrate that similar to the m3 mAChR, the stably transfected m2 mAChR and the endogenously expressed purinergic and LPA receptors can also induce a long-lasting G_i-mediated heterologous potentiation of PLC stimulation in HEK 293 cells and that PKC plays an essential role in this novel PLC regulatory process.

Experimental Procedures

Materials. *myo*-[^{3}H]Inositol (10–25 Ci/mmol) and D-*myo*-[^{3}H]Ins(1,4,5)P_3 (21 Ci/mmol) were from DuPont-New England Nuclear (Bad Homburg, Germany). [^{3}H]PtdIns(4,5)P_2 (1–5 Ci/mmol) was from Biotrend (Köln, Germany). Unlabeled D-*myo*-Ins(1,4,5)P_3 and staurosporine were from BIOMOL (Hamburg, Germany). Unlabeled phospholipids, LPA, and thrombin were from Sigma (Deisenhofen, Germany). Gö 6976 was from Calbiochem-Novabiochem (Bad Soden, Germany). All other materials were from previously described sources (Offermanns *et al*., 1994; Schmidt *et al*., 1995a, 1995b, 1996).

Cell culture. Culture conditions of HEK 293 cells stably expressing the human m2 or m3 mAChR subtypes with a density of 120,000 and 200,000 receptors/cell, respectively, were as reported in detail previously (Peralta *et al*., 1988; Schmidt *et al*., 1994). For experiments, cells subcultured in DMEM/F-12 medium were grown to near-confluence (175-cm^2 culture flasks and 145- or 35-mm culture dishes).

Agonist pretreatment and measurement of [^{3}H]inositol phosphate formation in intact cells. Cellular phosphoinositides were labeled by incubating nearly confluent monolayers of cells for 24 hr with *myo*-[^{3}H]inositol (0.5 μCi/ml) in growth medium. For PTX treatment, the cells were incubated during the last 16 hr of the labeling period with 100 ng/ml PTX. Next, the labeling medium was removed, and the adherent cells were equilibrated for 10 min at 37° in HBSS containing 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM D-glucose, buffered at pH 7.4 with 15 mM HEPES, and then incubated for 2 min at 37° in HBSS with and without receptor agonists in the absence of LiCl. Thereafter, the cell monolayers were washed 10 times with 1 ml each of agonist-free HBSS (37°) to remove free agonist. At the indicated periods of time, usually 30 min after the first agonist treatment, the adherent cells were incubated for 10 min at 37° with 10 mM LiCl in HBSS, followed immediately by the addition of stimulatory agents in the presence of 10 mM LiCl to measure formation of [^{3}H]inositol phosphates (30 min at 37°) or [^{3}H]Ins(1,4,5)P_3 (15 sec at 37°) as described previously (Schmidt *et al*., 1995b).

Measurement of [^{3}H]inositol phosphate formation in permeabilized cells. *myo*-[^{3}H]Inositol-prelabeled cell monolayers were treated for 2 min with and without 1 mM carbachol, followed by agonist washout (see above). At 30 min later, the medium was removed and replaced by 1 ml of assay buffer, containing 135 mM KCl, 5 mM $NaHCO_3$, 5 mM EGTA, 4 mM $MgCl_2$, 2 mM ATP, 1.5 mM $CaCl_2$ (corresponding to 40 nM free Ca^{2+}), 5.6 mM D-glucose, 10 mM LiCl, and 20 mM HEPES, pH 7.2. After 10 min at 37°, this buffer was replaced by fresh buffer containing an additional 10 μM digitonin without and with the stable GTP analog GTPγS (100 μM). Then the formation of [^{3}H]inositol phosphates was measured for 30 min at 37° (Schmidt *et al*., 1996).

Assay of PLC activity in HEK 293 cell lysates. Unlabeled HEK 293 cells were detached from the culture flasks, resuspended in HBSS, and treated for 2 min at 37° with and without 1 mM carbachol. Thereafter, the cells were washed free of agonist and homogenized, followed by measurement of PLC activity with exogenous [^{3}H]PtdIns(4,5)P_2 as described in detail previously (Camps *et al*., 1990; Schmidt *et al*., 1996).

Measurement of [^{3}H]phosphoinositides. [^{3}H]Phosphoinositides of HEK 293 cell monolayers prelabeled with *myo*-[^{3}H]inositol and treated for 2 min with receptor agonists, followed by agonist washout (see above), were measured exactly as described previously (Schmidt *et al*., 1996).

Determination of Ins(1,4,5)P_3 and PtdIns(4,5)P_2 masses. Unlabeled HEK 293 cells were treated for 2 min with and without receptor agonists, followed by agonist washout and, thereafter, 10-min treatment with 10 mM LiCl (see above). Then, the mass of PtdIns(4,5)P_2 was immediately determined or the adherent cells were incubated for 15 sec at 37° with and without receptor agonist to measure Ins(1,4,5)P_3 mass as described previously (Chilvers *et al*., 1991; Schmidt *et al*., 1996). Free Ins(1,4,5)P_3 and Ins(1,4,5)P_3 released from KOH-treated PtdIns(4,5)P_2 were measured by a radioreceptor assay (i.e., competition with binding of [^{3}H]Ins(1,4,5)P_3 to Ins(1,4,5)P_3-binding protein prepared from bovine adrenal cortex) (Chilvers *et al*., 1991).

Data presentation. Data shown are mean ± standard deviation from one experiment performed in triplicate and repeated as indicated. Results described in the text are mean ± standard error, with independent experiments performed in triplicate. Comparisons between mean values were made with the Student's paired *t* test or one-way analysis of variance, whichever was appropriate. A difference was regarded as significant at $p < 0.05$. Concentration-response curves were analyzed by fitting sigmoidal functions to the experimental data using iterative nonlinear regression analysis with the InPlot program (GraphPAD Software, San Diego, CA).

Results

Characteristics of receptor stimulation of PLC in m2 mAChR-expressing HEK 293 cells. Activation of m2 mAChRs stably expressed in HEK 293 cells causes a 2–4-fold increase in total [^{3}H]inositol phosphate formation. Half-maximal activation by carbachol is obtained at 10–20 μM (Schmidt *et al*., 1995b). PLC activity of HEK 293 cells is also stimulated by activation of endogenously expressed purinergic, LPA, and thrombin receptors (Fig. 1). Maximal ATP-stimulated PLC activation was up to ~3-fold, with half-maximal and maximal effects being observed at ~7 and ~100 μM ATP, respectively (Fig. 1; see also Fig. 4). Maximal LPA-stimulated PLC activation was up to ~2-fold, with an EC_{50} value of ~3 μM LPA (Fig. 1; see also Fig. 4). Thrombin

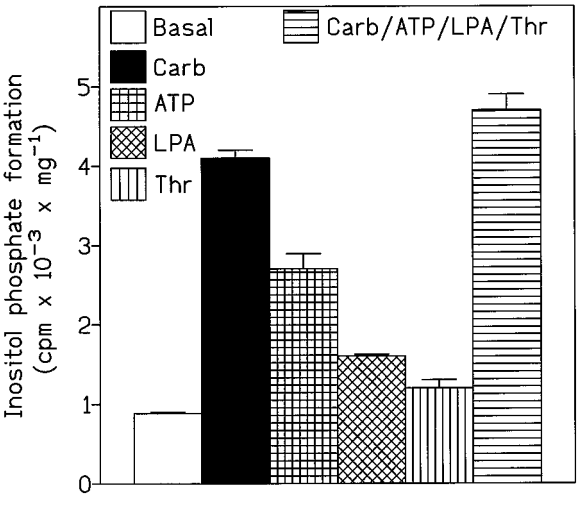

Fig. 1. Activation of PLC by different receptor agonists in m2 mAChR-expressing HEK 293 cells. Formation of [^{3}H]inositol phosphates was determined in *myo*-[^{3}H]inositol-prelabeled HEK 293 cells without (*Basal*) and with 1 mM carbachol (*Carb*), 1 mM ATP, 10 μM LPA, 4 units/ml thrombin (*Thr*), or a combination of all agonists (*Carb*/*ATP*/*LPA*/*Thr*) as indicated. Data are representative of three similar experiments.

increased PLC activity maximally by ~1.5-fold, with an EC_{50} value of ~2 units/ml (Fig. 1). Treatment of the m2 mAChR-expressing HEK 293 cells with PTX (100 ng/ml, 16 hr) had no effect on inositol phosphate formation stimulated briefly (up to 30 min) by either the transfected m2 mAChR (Offermanns *et al.*, 1994) or the endogenously expressed receptors (data not shown). Simultaneous addition of the different G protein-coupled receptor agonists, each at a maximally effective concentration, increased [^{3}H]inositol phosphate formation to a level that was not different from that induced by carbachol alone (Fig. 1).

m2 mAChR-induced heterologous potentiation of PLC stimulation. To study whether the rather poorly PLC-stimulating m2 mAChR can induce a long-lasting potentiation of PLC stimulation, as reported before for the highly efficient PLC-stimulating m3 mAChR (Schmidt *et al.*, 1995a, 1996), the m2 mAChR-expressing HEK 293 were treated for 2 min with carbachol, followed by agonist washout and, ≥40 min later, measurement of basal and agonist-stimulated [^{3}H]inositol phosphate formation. As shown in Fig. 2, the m2 mAChR was equally capable to induce this potentiation as the m3 subtype. At 40 min after the 2-min pretreatment of m2 mAChR-expressing HEK 293 cells with 1 mM carbachol, [^{3}H]inositol phosphate accumulation induced by a rechallenge of the cells with 1 mM carbachol was increased from 2.48 ± 0.08 to 3.98 ± 0.29 × 10^3 cpm/mg of protein (five experiments, $p < 0.001$) (Fig. 2A, *top*). Time course studies indicated that the relative enhancement of [^{3}H]inositol phosphate formation in carbachol-pretreated cells was similar at 5–30 min of incubation (data not shown). Short term carbachol treatment (1 mM, 2 min) of m2 mAChR-expressing HEK 293 cells not only induced a homologous sensitization of mAChR-mediated PLC stimulation but also largely potentiated purinergic receptor-induced PLC stimulation (Fig. 2A, *bottom*). [^{3}H]Inositol phosphate formation induced by 1 mM ATP in control and carbachol-pretreated cells amounted to 2.35 ± 0.63 and 5.23 ± 0.32 × 10^3 cpm/mg of protein, respectively (five experiments, $p < 0.001$). Furthermore, the m2 mAChR-induced potentiation of carbachol- and ATP-induced PLC stimulation, which was not accompanied by an increased basal [^{3}H]inositol phosphate formation, was observed for up to ~90 min after agonist removal (Fig. 2A). The carbachol-induced PLC potentiation was fully prevented when carbachol pretreatment was performed in the presence of the mAChR antagonist atropine (10 μM) (data not shown). The m2 mAChR-induced heterologous sensitization of PLC stimulation was not restricted to the endogenously expressed purinergic receptors. Carbachol treatment (1 mM, 2 min) also markedly enhanced PLC stimulation by LPA (10 μM) and thrombin (4 units/ml), measured 40 min after washout of carbachol (Fig. 2B). The LPA-induced [^{3}H]inositol phosphate accumulation was increased from 1.52 ± 0.05 to 2.90 ± 0.26 × 10^3 cpm/mg of protein (four experiments, $p < 0.002$), and that induced by thrombin was increased from 1.78 ± 0.10 to 6.75 ± 0.13 × 10^3 cpm/mg of protein (five experiments, $p < 0.001$). Similar to the potentiation of the m2 mAChR and purinergic receptor responses, the heterologous sensitization of LPA and thrombin receptor-mediated PLC activation was long-lasting, persisting for up to ~90 min after carbachol washout (data not shown). Carbachol pretreatment also markedly increased the agonist-induced Ins(1,4,5)P_3 mass formation, measured 15 sec after agonist

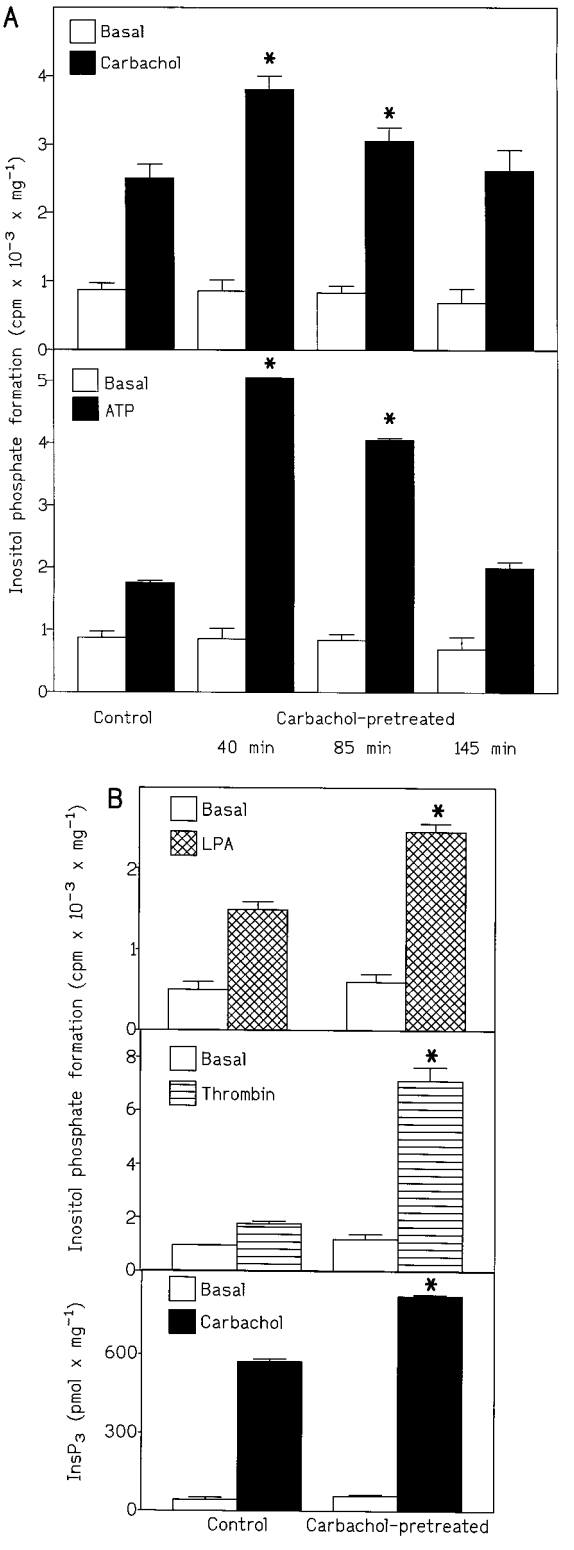

Fig. 2. m2 mAChR-induced long-lasting heterologous sensitization of receptor-mediated PLC responses. HEK 293 cell monolayers prelabeled or not with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) and with 1 mM carbachol (*Carbachol-pretreated*). A, At the indicated periods of time after washout of carbachol, formation of [^{3}H]inositol phosphates was determined without (*Basal*) and with 1 mM carbachol (top) or 1 mM ATP (*bottom*). B, At 40 min after carbachol washout, basal and LPA (10 μM, *top*)- or thrombin (4 units/ml, *middle*)-stimulated [^{3}H]inositol phosphate or carbachol (1 mM)-stimulated Ins(1,4,5)P_3 mass formation (*bottom*) was determined as described in the text. *, Significantly different ($p < 0.05$) from data for untreated controls.

addition, by ~40% (Fig. 2B, *bottom*). Ins(1,4,5)P_3 mass formation induced by ATP and LPA was similarly enhanced by carbachol pretreatment (data not shown). Thus, short term carbachol treatment of m2 mAChR-expressing HEK 293 cells can cause a long-lasting heterologous sensitization of receptor-mediated PLC responses.

Increasing carbachol concentrations during the pretreatment phase increased the sensitization of receptor-mediated PLC responses (Fig. 3). Although at 10 μM carbachol stimulation of PLC activities by LPA (10 μM) and ATP (1 mM) was not altered, m2 mAChR-mediated PLC stimulation was increased by ~1.5-fold. Pretreatment of HEK 293 cells for 2 min with 100 μM carbachol increased not only subsequent m2 mAChR-mediated PLC stimulation but also PLC stimulation by LPA and ATP by ~1.5-fold. At 1 mM carbachol during the pretreatment phase, the highest carbachol concentration examined, m2 mAChR-mediated PLC activation was potentiated by ~2-fold, and that induced by LPA and ATP was increased by ~2- and ~3-fold, respectively.

Shown in Fig. 4 are the concentration-response curves of carbachol, LPA, and ATP in control cells and HEK 293 cells 40 min after pretreatment for 2 min with 1 mM carbachol. Carbachol stimulated [^{3}H]inositol phosphate formation in control cells with an EC_{50} value of ~10 μM and a maximal stimulation at 100 μM (Fig. 4, *top*). In carbachol-pretreated cells, the carbachol concentration-response curve was shifted upward, with no major change in the slope of the curve. ATP stimulated [^{3}H]inositol phosphate production in control cells with an EC_{50} value of 6.7 μM and a maximal stimulation at 100 μM (Fig. 4, *middle*). In carbachol-pretreated cells, the EC_{50} value for ATP was increased by ~3-fold (19 μM), and maximal PLC activation was reached at 100 μM ATP. LPA stimulated [^{3}H]inositol phosphate accumulation in control cells with a rather steep concentration-response curve, with an EC_{50} value of 2.7 μM and a maximal stimulation at 10 μM (Fig. 4, *bottom*). In carbachol-pretreated cells, the EC_{50} value for LPA was slightly increased (3.3 μM), and maximal PLC stimulation was again reached at 10 μM LPA. Thus, overall, the m2 mAChR-induced potentiation of receptor-mediated PLC stimulation led to a general upward shift of the concentration-response curves of the receptor agonists, which was different in its extent but without large changes in the potency of the agonists.

In the m2 mAChR-expressing HEK 293 cells, PLC activity was also stimulated by direct activation of G proteins by AlF_4^- and the stable GTP analog GTPγS. Pretreatment of HEK 293 cells with carbachol markedly potentiated PLC stimulation by directly activated G proteins. As shown in Fig. 5, AlF_4^--induced [^{3}H]inositol phosphate formation in intact HEK 293 cells was ~2-fold higher in cells pretreated with 1 mM carbachol than in untreated controls (5.8 $\pm$ 0.17 versus 3.46 $\pm$ 0.23 $\times$ 10^3 cpm/mg of protein, three experiments, $p <$ 0.001). Similarly, GTPγS-induced [^{3}H]inositol phosphate formation in digitonin-permeabilized HEK 293 cells pretreated with carbachol was ~2-fold higher than in untreated control cells (Fig. 5). Carbachol pretreatment increased the GTPγS (100 μM)-stimulated [^{3}H]inositol phosphate formation from 1.30 $\pm$ 0.17 to 2.93 $\pm$ 0.11 $\times$ 10^3 cpm/mg of protein (three

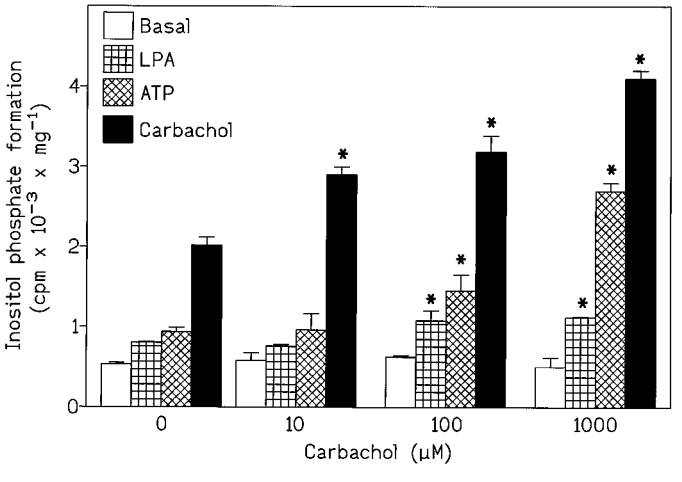

Fig. 3. Agonist concentration dependence of m2 mAChR-induced PLC potentiation. *myo*-[^{3}H]Inositol-prelabeled HEK 293 cells were treated for 2 min with the indicated concentrations of carbachol. At 40 min after carbachol washout, formation of [^{3}H]inositol phosphates was determined without (*Basal*) and with 10 μM LPA, 1 mM ATP, or 1 mM carbachol as indicated. Similar data were obtained in two independent experiments. *, Significantly different ($p < 0.05$) from data in cells not pretreated with carbachol.

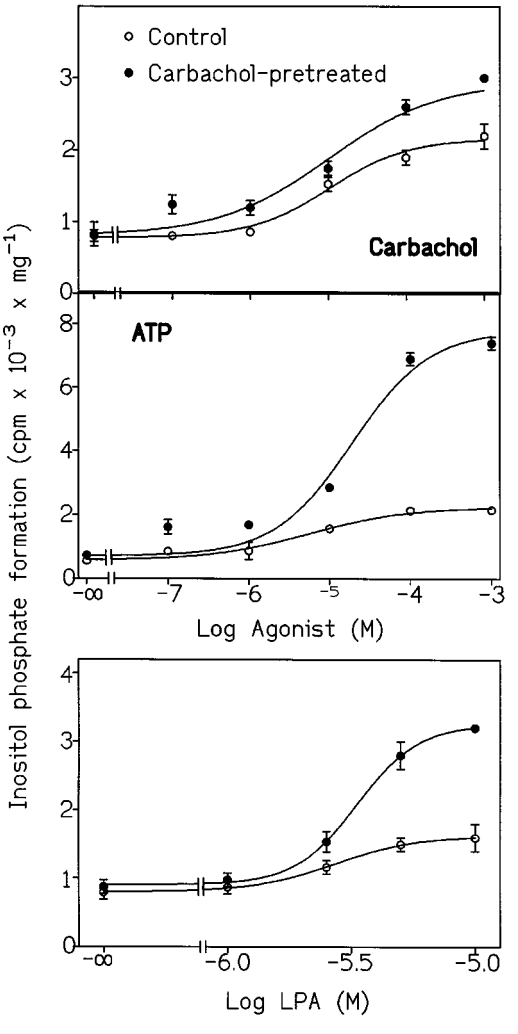

Fig. 4. Alteration in agonist concentration-response curves in carbachol-pretreated HEK 293 cells. m2 mAChR-expressing HEK 293 cells prelabeled with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) and with 1 mM carbachol (*Carbachol-pretreated*). At 40 min after carbachol washout, formation of [^{3}H]inositol phosphates was determined at the indicated concentrations of carbachol, ATP, or LPA. Similar data were obtained in at least two independent experiments.

experiments, $p < 0.001$). There also was a small but significant (three experiments, $p < 0.001$) increase in basal [^{3}H]inositol phosphate formation in carbachol-pretreated permeabilized HEK 293 cells, measured with 40 nM free Ca^{2+}, whereas in intact cells, basal accumulation of [^{3}H]inositol phosphates was not altered (see Figs. 2–4).

Heterologous potentiation of PLC stimulation induced by endogenously expressed receptors. To study whether potentiation of PLC stimulation is restricted to the stably transfected and overexpressed m2 and m3 mAChRs and, thus, whether activation of endogenously expressed G protein-coupled receptors may also induce sensitization of PLC stimulation, m2 mAChR-expressing HEK 293 cells were pretreated for 2 min with LPA (10 μM) or ATP (1 mM), followed by washout of the agonists and subsequent rechallenge with receptor agonists. Pretreatment of the cells with LPA and ATP did not induce a homologous PLC sensitization or desensitization, measured 40 and 70 min, respectively, after rechallenge with the respective receptor agonists (data not shown). However, as shown in Fig. 6, the m2 mAChR- and purinergic receptor-mediated PLC stimulation was potentiated in LPA-pretreated cells. LPA pretreatment increased carbachol (1 mM)- and ATP (1 mM)-induced [^{3}H]inositol phosphate formation from 7.75 ± 0.54 to 12.0 ± 1.08 × 10^3 cpm/mg of protein (four experiments, $p < 0.001$) and from 3.67 ± 0.32 to 5.04 ± 0.20 × 10^3 cpm/mg of protein (four experiments, $p < 0.04$), respectively. Furthermore, the carbachol-stimulated [^{3}H]inositol phosphate formation was increased from 2.68 ± 0.25 × 10^3 cpm/mg of protein in control cells to 4.6 ± 0.72 × 10^3 cpm/mg of protein in ATP-pretreated cells (three experiments, $p < 0.04$) (Fig. 6). The potentiation of the carbachol response in ATP-pretreated cells was observed only when the interval between the ATP treatment and the rechallenge with carbachol was increased from 40 to 70 min (data not shown).

Receptor-induced increase in cellular PtdIns(4,5)P_2. To study whether potentiation of PLC stimulation induced by the transfected m2 mAChR and the endogenously expressed purinergic and LPA receptors is accompanied by an increase in the cellular level of PtdIns(4,5)P_2, as reported previously for the m3 mAChR (Schmidt *et al.*, 1996), *myo*-[^{3}H]inositol-prelabeled HEK 293 cells were treated for 2 min without and with carbachol (1 mM), ATP (1 mM), or LPA (10 μM). After 40 min (70 min for ATP), the labeled phosphoinositides were extracted and quantified (i.e., exactly at the same time points and conditions as used for measurement of inositol phosphate formation). Short term pretreatment of HEK 293 cells with any of the three agonists had no effect on the levels of [^{3}H]PtdIns and [^{3}H]PtdIns4P, measured 40 min (70 min) after agonist removal (data not shown). However, the carbachol pretreatment significantly increased, by 55.4 ± 5.2% (three experiments, $p < 0.001$), the level of [^{3}H]PtdIns(4,5)P_2 in HEK 293 cells, compared with untreated controls (Fig. 7, *left*). Pretreatment of HEK 293 cells with ATP and LPA caused a similar increase in the cellular level of [^{3}H]PtdIns(4,5)P_2, by 38.9 ± 2.5% (three experiments, $p < 0.001$) and 58.9 ± 6.2% (three experiments, $p < 0.001$), respectively. Likewise, as shown in Fig. 7 (*right*), PtdIns(4,5)P_2 mass was elevated from 107.9 ± 12.8 pmol/mg of protein in control cells to 224.7 ± 21.1 pmol/mg of protein (six experiments, $p < 0.001$) in carbachol-pretreated HEK 293 cells. ATP and LPA pretreatment also enhanced PtdIns(4,5)P_2 mass (data not shown).

Role of G_i proteins in m2 mAChR-induced PLC potentiation. m2 mAChRs, although inducing PLC stimulation in HEK 293 cells in a PTX-insensitive manner, primarily couple to G_i-type G proteins in these and other cell types (Hulme *et al.*, 1990; Offermanns *et al.*, 1994). To study whether this m2 mAChR-G_i protein coupling is involved in PLC potentiation, the m2 mAChR-expressing HEK cells were pretreated with PTX (100 ng/ml, 16 hr) before the short term (2 min) treatment with carbachol (1 mM). As reported previously (Offermanns *et al.*, 1994; Schmidt *et al.*, 1995b), PTX treatment had no significant effect on the carbachol- and ATP-stimulated [^{3}H]inositol phosphate formation in control cells (Fig. 8). However, the carbachol-induced sensitization of PLC stimulation by carbachol and ATP was completely prevented by prior PTX treatment. In PTX-treated HEK 293 cells, pretreated for 2 min without and with 1 mM carbachol, carbachol (1 mM) increased [^{3}H]inositol phosphate formation by 1.9 ± 0.15 and 1.8 ± 0.2 × 10^3 cpm/mg of protein, respectively, and that induced by ATP (1 mM) amounted to 0.73 ± 0.1 and 0.72 ± 0.2 × 10^3 cpm/mg of protein, respectively (three experiments). Furthermore, PTX treatment, which did not affect the levels of [^{3}H]PtdIns and [^{3}H]PtdIns4P, reduced the carbachol-induced increase in the level of [^{3}H]PtdIns(4,5)P_2, from a 55 ± 2% increase in nonintoxicated cells to a 29 ± 5% (three experiments, $p < 0.001$) increase in PTX-pretreated cells. The ATP- and LPA-induced elevations in [^{3}H]PtdIns(4,5)P_2 were similarly reduced by PTX treatment, from 35 ± 2.3% to 24.1 ± 4% (three experiments, $p < 0.001$) and from 58 ± 5.2% to 27.7 ± 5.2% (three experiments,

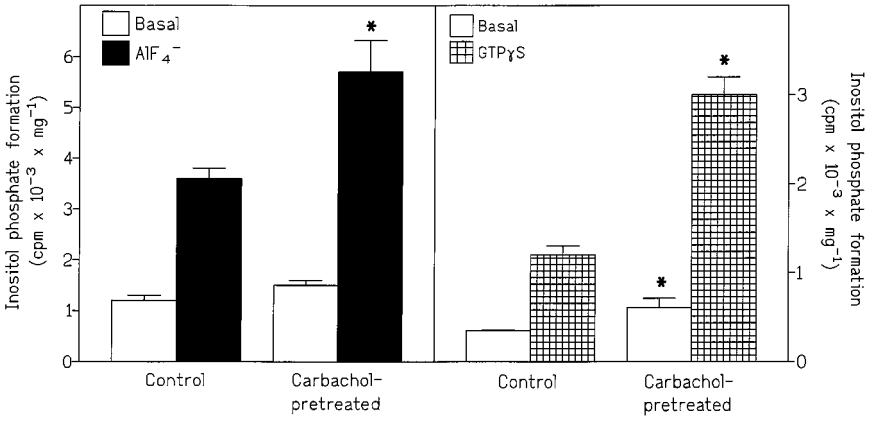

Fig. 5. Sensitization of G protein-mediated PLC stimulation. m2 mAChR-expressing HEK 293 cells prelabeled with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) and with 1 mM carbachol (*Carbachol-pretreated*). *Left*, at 40 min after washout of carbachol, formation of [^{3}H]inositol phosphates was determined in intact cells without (*Basal*) and with AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$). *Right*, at 40 min after washout of carbachol, formation of [^{3}H]inositol phosphates was determined in digitonin-permeabilized cells without (*Basal*) and with 100 μM GTPγS. *, Significantly different ($p < 0.05$) from data for untreated controls.

$p < 0.001$), respectively (data not shown). However, the levels of [^{3}H]PtdIns(4,5)P_2 were still significantly ($p < 0.05$) increased by any of the three agonists in PTX-treated cells.

Role of PKC in mAChR-induced PLC potentiation. The PKC enzymes represent one of the major downstream effectors of PLC signaling (Berridge, 1993; Nishizuka, 1995). Because PKC may modulate both acute PLC stimulation and its potentiation, we examined the effects of direct PKC activation by the phorbol ester PMA and of the PKC inhibitors staurosporine and Gö 6976 on agonist-induced PLC stimulation in control and carbachol-pretreated cells. For this, m2 mAChR-expressing HEK 293 cells were first treated without and with PMA (100 nM, 10 min) or the two PKC inhibitors (100 nM each, 30 min). Under these conditions, PMA induced a large increase in phospholipase D activity in HEK 293 cells, which was totally abolished by the two PKC inhibitors (Schmidt *et al.*, 1994; data not shown). Thereafter, the cells were treated for 2 min without and with 1 mM carbachol; 40 min later, [^{3}H]inositol phosphate formation induced by carbachol (1 mM) and ATP (1 mM) was monitored. PMA treatment had no effect on carbachol- and ATP-induced PLC stimulation in control cells (Fig. 9). In addition, PMA treatment did not affect the potentiation of PLC stimulation by carbachol and ATP in carbachol-pretreated HEK 293 cells. In PMA-treated HEK 293 cells, carbachol-stimulated [^{3}H]inositol phosphate formation in control and carbachol-pretreated cells amounted to 5.8 ± 0.2 and $8.7 \pm 0.15 \times 10^3$ cpm/mg of protein (three experiments, $p < 0.001$), respectively, and that induced by ATP to 3.1 ± 0.1 and $5.8 \pm 0.2 \times 10^3$ cpm/mg of protein (three experiments, $p < 0.002$), respectively.

In contrast to PMA, the treatment of HEK 293 cells with the PKC inhibitors staurosporine and Gö 6976, which did not affect acute PLC stimulation by either carbachol or ATP, completely abolished the carbachol-induced potentiation of PLC stimulation in m2 mAChR-expressing HEK 293 cells (Fig. 10). In cells treated with staurosporine, carbachol (1 mM) increased [^{3}H]inositol phosphate production in control and carbachol-pretreated cells by 4.5 ± 0.5 and $5.2 \pm 0.32 \times 10^3$ cpm/mg of protein, respectively, and that induced by ATP (1 mM) amounted to 3.5 ± 0.1 and $3.1 \pm 0.2 \times 10^3$ cpm/mg of protein, respectively. In Gö 6976-treated HEK 293 cells, carbachol (1 mM) increased [^{3}H]inositol phosphate formation in control and carbachol-pretreated cells by 5.9 ± 0.4 and $6.2 \pm 0.12 \times 10^3$ cpm/mg of protein (three experiments), respectively, and that induced by ATP (1 mM) was 3.0 ± 0.2 and $2.9 \pm 0.4 \times 10^3$ cpm/mg of protein (three experiments), respectively. The m3 mAChR-induced potentiation of PLC stimulation exhibited a similar sensitivity to the PKC inhibitors (Fig. 11). Carbachol (1 mM) increased [^{3}H]inositol phosphate production in control and carbachol-pretreated (1 mM, 2 min) cells by 11.8 ± 0.86 and $20.0 \pm 1.9 \times 10^3$ cpm/mg of protein (six experiments, $p < 0.001$), respectively, and ATP (1 mM) by 1.34 ± 0.15 and $3.52 \pm 0.29 \times 10^3$ cpm/mg of protein (six experiments, $p < 0.001$), respectively. In cells pretreated with Gö 6976 (100 nM, 30 min), the carbachol-induced increase in [^{3}H]inositol phosphate production in control and carbachol-pretreated cells amounted to 10.8 ± 0.26 and $11.4 \pm 0.63 \times 10^3$ cpm/mg of protein (six experiments), respectively, and that induced by ATP to 1.57 ± 0.10 and $1.63 \pm 0.05 \times 10^3$ cpm/mg of protein (six experiments), respectively. Similar data were obtained in m3 mAChR-expressing HEK 293 cells treated with staurosporine (data not shown).

As both PTX and the PKC inhibitors prevented receptor-induced potentiation of PLC stimulation and as PTX reduced the increase in cellular [^{3}H]PtdIns(4,5)P_2 level, we studied whether the PKC inhibitors may have a similar effect on the level of the PLC substrate. As shown in Fig. 12, treatment of m2 mAChR-expressing HEK 293 cells with Gö 6979 (100 nM) for 30 min significantly ($p < 0.02$, three experiments) reduced the carbachol-induced increase in the level of [^{3}H]PtdIns(4,5)P_2, from $63 \pm 2.0\%$ to $32 \pm 2.1\%$, similar as observed with PTX (reduction to $24 \pm 1.2\%$, $p < 0.01$). Neither treatment alone caused a complete blockade of the carbachol-induced elevation in [^{3}H]PtdIns(4,5)P_2 level, which, however, was obtained by treatment of the cells with both PTX and Gö 6976. Similar results on agonist-induced in-

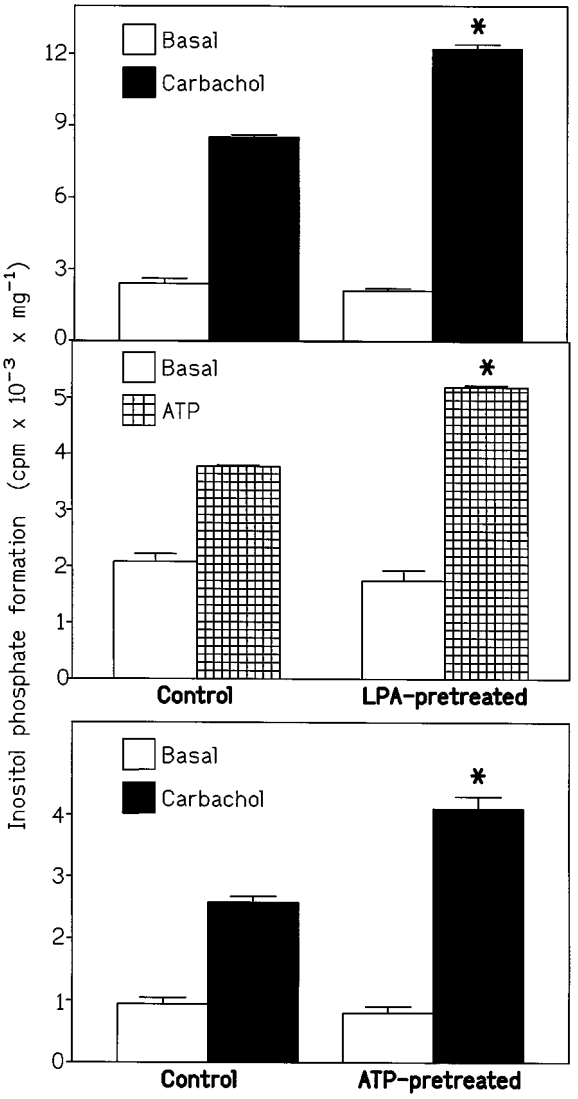

Fig. 6. Potentiation of PLC stimulation by endogenously expressed G protein-coupled receptors. m2 mAChR-expressing HEK cells prelabeled with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) and with 10 μM LPA (*LPA-pretreated*) or 1 mM ATP (*ATP-pretreated*). At 40 min after LPA washout and 70 min after ATP washout, formation of [^{3}H]inositol phosphates was determined without (*Basal*) and with 1 mM carbachol or 1 mM ATP as indicated. *, Significantly different ($p < 0.05$) from data in untreated controls.

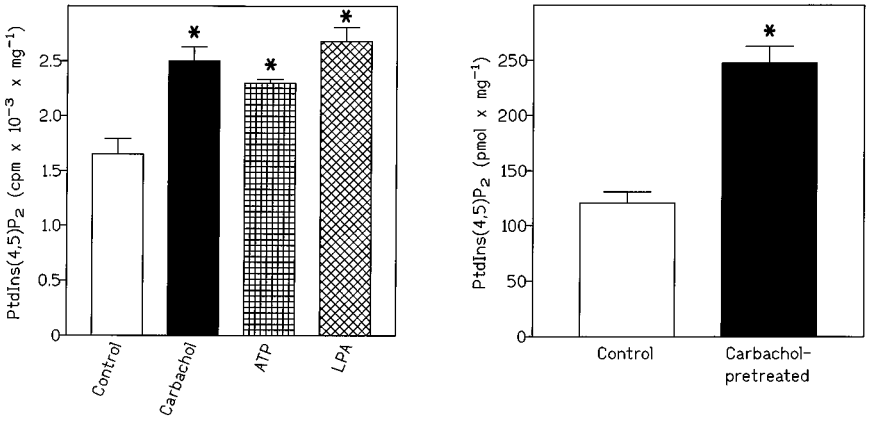

Fig. 7. Influence of agonist pretreatment on the level of PtdIns(4,5)P_2 in HEK 293 cells. *Left*, level of [^{3}H]phosphoinositides was determined in m2 mAChR-expressing HEK 293 cells prelabeled with *myo*-[^{3}H]inositol and subsequently treated for 2 min without (*Control*) and with 1 mM carbachol, 1 mM ATP, or 10 μM LPA. At 40 min after carbachol and LPA washout or 70 min after ATP washout, the levels of [^{3}H]PtdIns, [^{3}H]PtdIns4P, and [^{3}H]PtdIns(4,5)P_2 were quantified. The levels of [^{3}H]PtdIns and [^{3}H]PtdIns4P were 34.9 ± 1.6 and 0.78 ± 0.2 × 10^3 cpm/mg of protein, respectively, in untreated control cells and were not changed by agonist treatment (data not shown). *Right*, at 40 min after carbachol washout, PtdIns(4,5)P_2 mass was determined in unlabeled HEK 293 cells. *, Significantly different ($p < 0.01$) from untreated controls.

crease in cellular [^{3}H]PtdIns(4,5)P_2 level were obtained with staurosporine (data not shown).

Discussion

Receptor-mediated PLC stimulation, resulting in formation of the two second messengers, Ins(1,4,5)P_3 and diacylglycerol, which control intracellular Ca^{2+} levels and activity of PKC enzymes, respectively, initiates a large variety of early and late cellular responses, such as secretion, cell growth, smooth muscle contraction, and neuronal signaling (Berridge, 1993; Nishizuka, 1995; Rhee and Bae, 1997). Thus, a crucial impact of potentiated PLC-dependent signaling on various cellular functions can be expected. Indeed, long term potentiation or sensitization, induced by PLC stimulatory receptors and G proteins, of various cellular functions has been described in several neuronal and peripheral cellular systems (Gosh and Greenberg, 1995; Auerbach and Segal, 1996; Gong *et al.*, 1996). However, the exact mechanisms leading to such phenomena have not been identified yet.

We recently reported that short term agonist treatment of m3 mAChR-expressing HEK 293 cells can induce a huge and long-lasting sensitization of PLC stimulation (Schmidt *et al.*, 1995a). In subsequent analysis of this unexpected finding, we could demonstrate the heterologous nature of this potentiated receptor response as well as an involvement of PTX-sensitive G proteins (Schmidt *et al.*, 1996). In the current study, we report that this long-lasting heterologous PLC potentiation is not restricted to the highly efficient PLC-stimulating m3 subtype of mAChRs. Short term agonist activation of the m2 mAChR, stably transfected in HEK 293 cells and inducing by itself only a very modest PLC stimulation compared with that induced by the m3 mAChR (Peralta *et al.*, 1988; Offermanns *et al.*, 1994; Schmidt *et al.*, 1995b), can induce a similar potentiation of PLC stimulation. The m2 mAChR-induced potentiation was heterologous and long-lasting, persisting for up to ~90 min after carbachol removal. Carbachol pretreatment of m2 mAChR-expressing HEK 293 cells not only increased PLC stimulation by carbachol itself but also that induced by ATP, LPA, and thrombin, ligands for receptors endogenously expressed in HEK 293 cells, as well as PLC stimulation by directly activated G proteins in intact (by AlF_4^-) and permeabilized HEK 293 cells (by GTPγS).

Most important, we demonstrate here that heterologous potentiation of PLC stimulation is not restricted to the transfected and overexpressed mAChRs but can also be induced by activation of purinergic ($P2_Y$ subtype) and LPA receptors

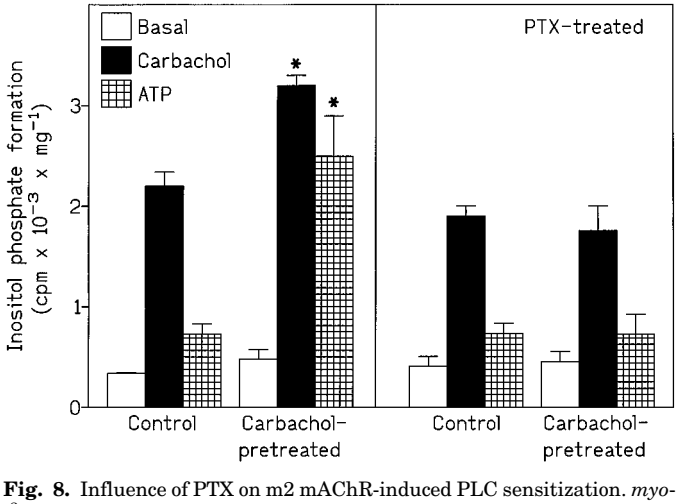

Fig. 8. Influence of PTX on m2 mAChR-induced PLC sensitization. *myo*-[^{3}H]Inositol-prelabeled HEK 293 cells were pretreated for 16 hr without (*left*) and with 100 ng/ml PTX (*right*) and then for 2 min without (*Control*) and with 1 mM carbachol (*Carbachol-pretreated*). After 40 min, [^{3}H]inositol phosphate formation was determined without (*Basal*) and with 1 mM carbachol or 1 mM ATP. *, Significantly different ($p < 0.05$) from cells not treated with carbachol.

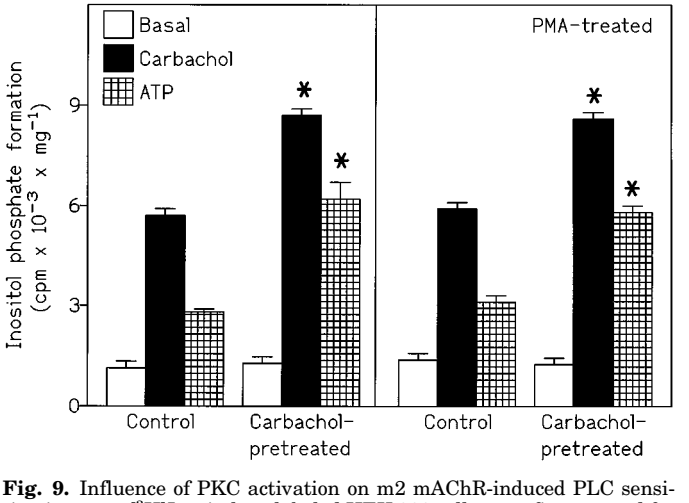

Fig. 9. Influence of PKC activation on m2 mAChR-induced PLC sensitization. *myo*-[^{3}H]Inositol-prelabeled HEK 293 cells were first treated for 10 min with 100 nM PMA (*right*) or its solvent, dimethylsulfoxide (0.1%, v/v, *left*). Immediately thereafter, the cells were treated for 2 min without (*Control*) and with 1 mM carbachol (*Carbachol-pretreated*). After 40 min, [^{3}H]inositol phosphate formation was determined without (*Basal*) and with 1 mM carbachol or 1 mM ATP. *, Significantly different ($p < 0.05$) from cells not treated with carbachol.

endogenously expressed in HEK 293 cells. Compared with the overexpressed m3 and m2 mAChRs, acute PLC stimulation by these endogenously expressed receptors was very minor. Nevertheless, short term pretreatment of HEK 293 cells with LPA induced a potentiation of carbachol- and ATP-induced inositol phosphate formation, and a similar increased carbachol response was observed in ATP-pretreated cells. The extent of PLC potentiation was similar, regardless of whether potentiation was induced by the overexpressed mAChRs or the endogenously expressed purinergic and LPA receptors, suggesting that the extent of acute PLC stimulation *per se* does not dictate the extent of PLC potentiation. Under the conditions studied, no homologous potentiation of PLC stimulation was observed in ATP- or LPA-pretreated cells. This may be due to a loss of cell surface receptors and/or a prolonged desensitization of PLC stimulation, masking a potential homologous PLC potentiation and, thus, resulting in an apparently unaltered PLC response in the pretreated cells. Receptor-induced potentiation of PLC stimulation seems not to be an acute cellular response. This became most obvious when we studied PLC potentiation by ATP. Although in cells pretreated for 2 min with carbachol or LPA potentiation of PLC stimulation was observed 40 min after agonist removal, the interval between the first and the second agonist treatment had to be increased to 70 min to observe the ATP-induced PLC potentiation. Thus, in contrast to desensitization of PLC stimulation by G protein-coupled receptors, which is a rather early cellular reaction (Wojcikiewicz *et al.*, 1993; Fischer, 1995), potentiation of PLC stimulation, although requiring only a short initial stimulus, apparently is a rather late cellular response that then lasts for a long period of time.

Receptor-induced PLC potentiation in HEK 293 cells obviously involves a G_i protein-dependent process. The m3 mAChR preferentially couples to G_q-type G proteins, with G_i

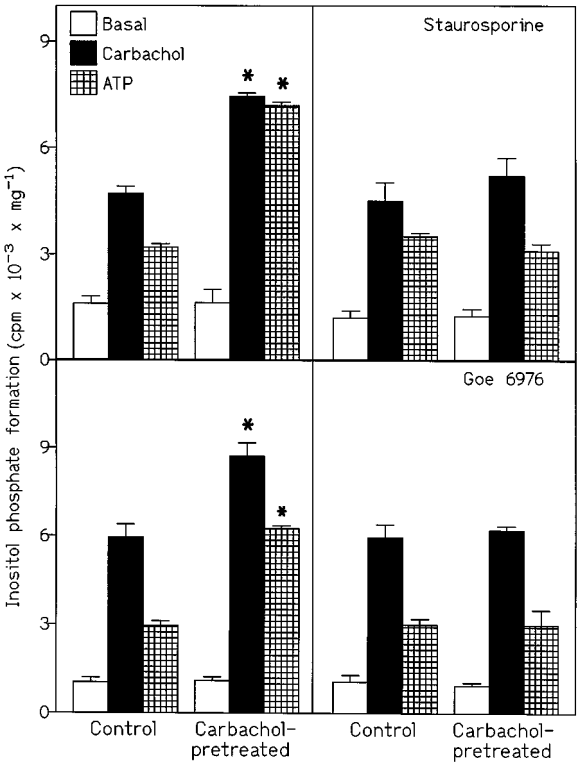

Fig. 10. Influence of PKC inhibition on m2 mAChR-induced PLC sensitization. *myo*-[^{3}H]Inositol-prelabeled HEK 293 cells were first treated for 30 min with 100 nM staurosporine (*top right*), 100 nM Gö 6976 (*bottom right*) or their solvent, dimethylsulfoxide (0.1%, v/v, *left*). Immediately thereafter, the cells were treated for 2 min without (*Control*) and with 1 mM carbachol (*Carbachol-pretreated*). After 40 min, [^{3}H]inositol phosphate formation was determined without (*Basal*) and with 1 mM carbachol or 1 mM ATP. In cells pretreated with staurosporine or Gö 6976 (*Goe 6976*), these agents were also present during carbachol treatment and in the PLC assays. *, Significantly different ($p < 0.05$) from cells not treated with carbachol.

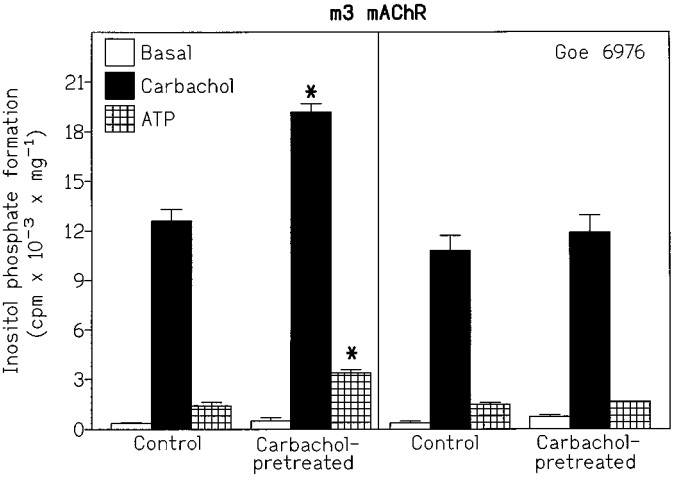

Fig. 11. Influence of PKC inhibition on m3 mAChR-induced PLC sensitization. Identical protocol as in Fig. 10, except that the effect of Gö 6976 was studied on m3 mAChR-expressing HEK 293 cells. *, Significantly different ($p < 0.05$) from cells not treated with carbachol.

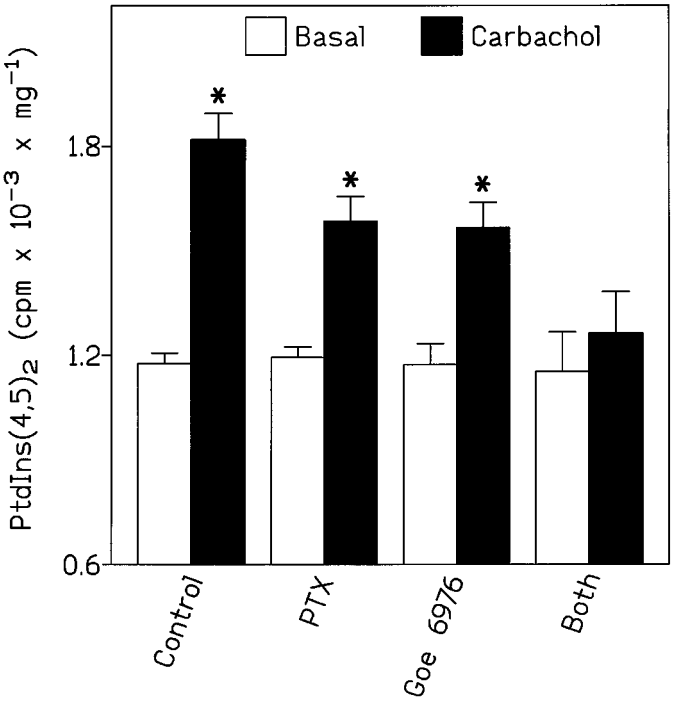

Fig. 12. Influence of PTX and PKC inhibition on m2 mAChR-induced elevation in cellular PtdIns(4,5)P_2. Treatment of HEK 293 cells with PTX and Gö 6976 (100 nM) was as described in legends to Fig. 8 and 10, respectively, and measurement of [^{3}H]PtdIns(4,5)P_2 in control and carbachol-pretreated (1 mM, 2 min) cells was determined as described in legend to Fig. 7. The levels of [^{3}H]PtdIns and [^{3}H]PtdIns4P (not shown) were 21.9 ± 0.8 and 0.56 ± 0.10 × 10^3 cpm/mg of protein, respectively, in control cells and were not affected by either PTX or Gö 6976.

protein activation being observed only at high agonist concentrations (Offermanns *et al*., 1994). In contrast, the m2 mAChR is known to couple to and activate G_i-type G proteins with high potency in HEK 293 and other cell types (Hulme *et al*., 1990; Offermanns *et al*., 1994). However, apparently regardless of the preferential G protein-coupling selectivity of m2 and m3 mAChRs, PTX treatment of HEK 293 cells completely prevented the potentiation of PLC stimulation induced by either mAChR subtype. These data indicate that G_i proteins, apparently not involved in acute PLC stimulation in HEK 293 cells, control one essential mechanism involved in receptor-induced PLC potentiation.

The G_i protein-controlled pathway apparently is not the sole cellular mechanism involved in receptor-induced PLC potentiation. Although the m2 mAChR activates with high potency G_i proteins in HEK 293 cells (Offermanns *et al*., 1994), potentiation of PLC stimulation was observed only at rather high carbachol concentrations, very similar to those required for acute PLC stimulation (Schmidt *et al*., 1995b). These data suggested that PLC stimulation and/or a consequence of this stimulation is necessary for receptor-induced PLC potentiation. Because activation of PKC is one of the major consequences of PLC signaling (Berridge, 1993; Nishizuka, 1995), we studied the effects of the phorbol ester PMA, a direct activator of PKC enzymes, and of staurosporine and Gö 6976, two distinct PKC inhibitors, on PLC stimulation in HEK 293 cells and its potentiation by agonist-activated mAChRs. In contrast to various other receptor and cell types, for which an inhibitory effect of acute PMA treatment on receptor-mediated PLC signaling has been reported (Della Bianca *et al*., 1986; Brown *et al*., 1987; Vázquez-Prado and García-Sáinz, 1996; Kawabata *et al*., 1996), short term treatment of HEK 293 cells with PMA had no effect on acute agonist-induced PLC stimulation. Furthermore, m2 mAChR-induced potentiation of PLC stimulation was not altered, neither decreased nor increased, after acute PMA treatment. In apparent contrast, Hepler *et al*. (1988) and Chen *et al*. (1995) reported that PMA treatment can increase receptor-induced PLC stimulation. However, in these studies, the cells were treated on a long term basis with PMA to down-regulate PKC enzymes. Long term treatment of HEK 293 cells with PMA (100 nM, 16 hr) caused a reduction rather than an increase in PLC stimulation, which may be due to decreased phosphoinositide levels observed in chronically but not acutely PMA-treated cells (data not shown). On the other hand, pretreatment of HEK 293 cells with the PKC inhibitors staurosporine and Gö 6976, which did not affect acute PLC stimulation, completely abolished PLC potentiation induced by either m2 or m3 mAChR subtypes. Thus, in addition to G_i proteins, activation of a PKC enzyme or enzymes is apparently essential for mAChR-induced potentiation of PLC stimulation in HEK 293 cells. Because acute PLC as well as phospholipase D stimulation by mAChRs in HEK 293 cells is PTX insensitive (Offermanns *et al*., 1994; Schmidt *et al*., 1994), it is most likely that the PKC enzyme or enzymes apparently involved in PLC potentiation are not under the control of G_i proteins, further suggesting that two pathways, one controlled by G_i proteins and one controlled by PKC, are involved in mAChR-induced potentiation of PLC stimulation.

Receptor-mediated PLC potentiation was accompanied by an enhanced cellular level of PtdIns(4,5)P_2 (Schmidt *et al*., 1996; Fig. 7). Furthermore, using exogenous PtdIns(4,5)P_2 to measure PLC activity in lysates of HEK 293 cells pretreated or not with carbachol, no differences in PLC activities stimulated by either directly activated G proteins (AlF_4^-, GTPγS) or Ca^{2+} were noted (Schmidt *et al*., 1996; data not shown). These data suggested that the agonist-induced increase in the PLC substrate level is involved in PLC potentiation. On the other hand, treatment of HEK 293 cells with PTX or the PKC inhibitors fully prevented the receptor-induced PLC potentiation but reduced the receptor-induced increase in PtdIns(4,5)P_2 level by only ~50%. A complete blockade of this response was achieved only in cells treated with both PTX and PKC inhibitors. Thus, because only total cellular PtdIns(4,5)P_2 levels were measured, it must be studied whether there are distinct cellular pools of PtdIns(4,5)P_2, whether these pools are distinctly regulated by PTX-sensitive G proteins and PKC enzymes, and, most important, which of these PtdIns(4,5)P_2 pools is accessible to receptor-induced PLC stimulation.

In conclusion, the data presented in this report indicate that activation of m2 mAChRs in HEK 293 cells can induce a long-lasting G_i-mediated heterologous potentiation of receptor signaling to PLC, similar to that reported previously for the m3 mAChR, although these two mAChR subtypes exhibit distinct preferential G protein coupling and distinct PLC stimulation efficiencies. Most important, receptor-induced heterologous PLC potentiation was not restricted to the stably transfected and overexpressed m2 and m3 mAChRs but was also observed on activation of endogenously expressed purinergic and LPA receptors, suggesting that agonist-induced potentiation of PLC stimulation is a rather general phenomenon. The data presented further suggest that in addition to G_i proteins, activation of a PKC enzyme or enzymes is an essential prerequisite of this novel PLC regulatory process. Because the products of PLC-catalyzed hydrolysis of PtdIns(4,5)P_2, Ins(1,4,5)P_3 and diacylglycerol control a variety of early and late cellular processes, it is thus tempting to speculate that the receptor-induced long term potentiation of PLC signaling may have a major impact on various cellular functions, known to be under PLC control.

Acknowledgments

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