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m3 Muscarinic Receptor-Induced and G_i-Mediated Heterologous Potentiation of Phospholipase C Stimulation: Role of Phosphoinositide Synthesis

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SUMMARY

Agonist activation of thrombin and purinergic receptors endogenously expressed in human embryonic kidney (HEK) cells and of the stably expressed m3 muscarinic acetylcholine receptor (mAChR) induces phospholipase C (PLC) stimulation, with the most pronounced PLC stimulation observed on mAChR activation. These receptor responses were pertussis toxin (PTX) insensitive and nonadditive, suggesting that the receptors share common signaling pathways. Short term (2 min) pretreatment of HEK cells with carbachol (1 mM), but not ATP, followed by agonist washout, caused a long-lasting (≥90 min) sensitization of PLC responses. At 30 min after carbachol treatment and washout, mAChR-stimulated PLC activity, measured as formation of either total inositol phosphates or of inositol-1,4,5-trisphosphate, was enhanced by 1.5–2-fold. PLC stimulation by thrombin and purinergic receptors was increased by ~3-fold. Furthermore, carbachol pretreatment also enhanced, by ~2.5-fold, stimulation of PLC activity on direct activation of G proteins by AlF_4^- and guanosine-5′-O-(3-thio)-triphosphate in intact and permeabilized cells, respectively. In contrast, PLC activities, measured with exogenous phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] in HEK cell lysates, were not altered, suggesting that carbachol pretreatment may enhance the cellular level of PtdIns(4,5)P_2. Indeed, the level of PtdIns(4,5)P_2 was found to be increased by ~50% in HEK cells 30 min after short term carbachol treatment, whereas the level of phosphatidylinositol was not altered and that of phosphatidylinositol-4-phosphate decreased (by 40–50%). Pretreatment of HEK cells with PTX prevented the m3 mAChR-induced PLC potentiation and reduced the elevation in PtdIns(4,5)P_2 level by ~50%. In conclusion, short term agonist activation of m3 mAChRs stably expressed in HEK cells can lead to a long-lasting heterologous potentiation of PLC signaling, which processes apparently involve PTX-sensitive G proteins and an enhanced PLC substrate supply.

Hydrolysis of the membrane phospholipid PtdIns(4,5)P_2 by PLC enzymes, generating the two second messenger molecules Ins(1,4,5)P_3 and diacylglycerol, is a ubiquitous response of eukaryotic cells downstream of various tyrosine kinase receptors and heptahelical receptors coupled to heterotrimeric G proteins (1–3). A large variety of hormones, neurotransmitters, and sensory signals stimulate PLC by activation of G protein-coupled receptors. These receptors activate PLC isoenzymes of the PLC-β subtype by two distinct mechanisms. The PTX-insensitive activation of PLC-β enzymes is apparently 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 βγ dimers of G_i-type G proteins. Furthermore, PLC-β isoenzymes (PLC-β1-3) display different sensitivities to GTP-liganded α_q subunits and free βγ dimers (4). Reconstitution of purified proteins supports the general assumption that only three components are required for PLC activation by G protein-coupled receptors (the receptor, a PLC-β isoenzyme, and the heterotrimeric G protein), with either the GTP-bound α subunit or the free βγ dimer being the actual PLC-activating component (4, 5).

In HEK cells stably expressing the human m3 mAChR subtype, the receptor activates $G_{q/11}$ proteins and efficiently stimulates PLC in a PTX-insensitive manner (6, 7). In studies on desensitization of phospholipase D stimulation by m3 mAChRs, we made the unexpected finding that short term agonist (carbachol) pretreatment of the cells causes a significant and long-lasting sensitization of m3 mAChR-mediated PLC activation (8). In the current study, we demonstrate that this short term carbachol pretreatment induces both a long-lasting sensitization of transfected mAChR responses and also a potentiation of PLC stimulation by endogenously ex-

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ABBREVIATIONS: PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; mAChR, muscarinic acetylcholine receptor; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol-4-monophosphate; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; PLC, phospholipase C; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

pressed thrombin and purinergic receptors as well as by directly activated G proteins, whereas PLC activity measured with exogenous PtdIns(4,5)P_2 was not affected. It is furthermore shown that carbachol treatment increases the cellular level of PtdIns(4,5)P_2 and that PTX treatment prevents sensitization of PLC stimulation and reduces by ~50% the elevation in PtdIns(4,5)P_2 level.

Experimental Procedures

Materials. *myo*-[^{3}H]Inositol (24.4 Ci/mmol) and [^{3}H]PtdIns(4,5)P_2 (1–5 Ci/mmol) were purchased from Biotrend (Koën, Germany), and D-*myo*-Ins(1,4,5)P_3 [^{3}H]assay system was from Amersham Buchler (Braunschweig, Germany). Unlabeled phosphoinositides, phosphatidylethanolamine, and thrombin were from Sigma Chemie (Deisenhofen, Germany). All other materials were from previously described sources (6–8).

Cell culture. Culture conditions of HEK cells stably expressing the human m3 mAChRs were as reported in detail before (7). 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).

Carbachol pretreatment and assay of PLC activity in intact cells. For measurement of intact cell PLC activity, cellular phospholipids were labeled by incubation of nearly confluent monolayers of cells for 24 hr with *myo*-[^{3}H]inositol (1 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. Later, 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, if not otherwise indicated, for 2 min at 37° in HBSS with and without 1 mM carbachol in the absence of LiCl. Thereafter, each of the cell monolayers were washed 10 times with 1 ml of agonist-free HBSS (37°) to remove free agonist. At the indicated periods of time (in most experiments shown, 30 min after agonist removal), the adherent cells were then incubated for 10 min at 37° with 10 mM LiCl in HBSS, followed immediately by the addition of carbachol or other stimulatory agents in the presence of 10 mM LiCl to measure the formation of [^{3}H]inositol phosphates (10 min at 37°) or [^{3}H]Ins(1,4,5)P_3 (15 sec at 37°) as described previously (7, 9).

In some experiments, *myo*-[^{3}H]inositol-prelabeled cells were detached from the culture flasks, centrifuged for 5 min at 500 × *g*, and resuspended in 25 ml of HBSS, followed by incubation for 2 min at 37° with and without 1 mM carbachol. Then, the cells were pelleted again (5 min at 500 × *g*) and resuspended in 25 ml of agonist-free HBSS. This centrifugation/resuspension procedure was repeated nine additional times to remove free agonist. At 60 min later, the cells were incubated for 10 min with 10 mM LiCl. Formation of [^{3}H]inositol phosphates or [^{3}H]Ins(1,4,5)P_3 was then measured in a total volume of 200 μl containing 1 × 10^6 cells, 10 mM LiCl, and the indicated stimulatory agents. The reaction was stopped by the addition of 2 ml of chloroform/methanol (1:1) and 1 ml of H_2O. After centrifugation for 10 min at 2000 × *g*, 1 ml of the upper aqueous phase was used for separation of [^{3}H]inositol phosphates and [^{3}H]Ins(1,4,5)P_3 (7, 9).

Assay of PLC activity in permeabilized adherent cells. Before permeabilization, *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 with 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 and the indicated stimulatory agents. Formation of [^{3}H]inositol phosphates was measured for 30 min at 37°.

Assay of PLC activity with exogenous substrate. Mixed phospholipid vesicles containing phosphatidylethanolamine and [^{3}H]PtdIns(4,5)P_2 in a molar ratio of 2:1 were dried and resuspended in 50 mM HEPES, 150 mM NaCl, and 2 mM sodium deoxycholate, pH 7.0, followed by sonication on ice (10). Unlabeled HEK cells were detached from the culture flasks, resuspended in HBSS, and treated for 2 min at 37° with and without 1 mM carbachol. Then, the cells were washed free of agonist by 10 centrifugations and resuspensions in excess HBSS (see above) and finally resuspended in 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, for homogenization with a glass-Teflon homogenizer. Assays were performed for 15 min at 37° in a total volume of 70 μl containing HEK cell lysate (5 μg of protein), 50 μM [^{3}H]PtdIns(4,5)P_2 (10,000 cpm), 100 μM phosphatidylethanolamine, 1 mM deoxycholate, and the indicated stimulatory agents in assay buffer. After the reactions were stopped and after phase separation, [^{3}H]inositol phosphate accumulation was determined in aliquots of the upper aqueous phase by liquid scintillation counting as described previously (10).

Analysis of phosphoinositides. HEK cell monolayers labeled with *myo*-[^{3}H]inositol were treated for 2 min with and without 1 mM carbachol, followed by agonist washout and 10-min treatment with 10 mM LiCl at 30 min later (see above). Then, 3 ml of 2.4 N HCl was added, and the cells (~6 × 10^7 cells, corresponding to ~20 mg of protein) were scraped from the culture dishes, followed by the addition of 10 ml of chloroform/methanol/concentrated HCl (200:100:0.75). After centrifugation for 10 min at 2000 × *g*, the lower phase containing the phosphoinositides was collected and evaporated, and the lipids were resuspended in 50 μl of chloroform/methanol (1:1). An aliquot was then applied onto oxalate-impregnated Silica Gel 60 plates, and the plates were developed in chloroform/methanol/2.5 N ammonium hydroxide (9:7:2) (11). Lipids were localized by iodine staining and identified by comigration with authentic standards. The areas corresponding to the phosphoinositides, PtdIns (R_f = 0.64), PtdIns4P (R_f = 0.45) and PtdIns(4,5)P_2 (R_f = 0.25), were scraped into scintillation vials, and the radioactivity was measured by liquid scintillation counting.

Determination of InsP_3 and PtdIns(4,5)P_2 masses. For measurement of Ins(1,4,5)P_3 and PtdIns(4,5)P_2 masses, unlabeled HEK cells were cultured in 35-mm culture dishes to near-confluence. Adherent cells were treated for 2 min with and without 1 mM carbachol, followed by agonist washout and 10-min treatment with 10 mM LiCl 30 min later (see above). Then, the incubation medium was removed, and 0.5 ml of ice-cold 0.5 M trichloroacetic acid was added to allow measurement of PtdIns(4,5)P_2 mass or the adherent cells were incubated for 15 sec at 37° with and without 1 mM carbachol, followed by the addition of trichloroacetic acid, to allow measurement of Ins(1,4,5)P_3 mass as described previously (12). Ins(1,4,5)P_3 purified by anion exchange columns (Amprep SAX, Amersham) was measured directly with the radioreceptor assay kit. For measurement of PtdIns(4,5)P_2 mass, phospholipids were first extracted and then treated with KOH, followed by measurement of Ins(1,4,5)P_3 mass, according to Chilvers *et al.* (12) and the manufacturer's instructions.

Data presentation. If not otherwise indicated, 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 each independent experiment performed in triplicate. Comparisons between mean values were made with the Student's paired *t* test. A difference was regarded as significant when at $p < 0.05$. The 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

PLC stimulation in m3 mAChR-expressing HEK cells. Agonist activation of m3 mAChRs stably expressed in HEK cells causes a large increase in inositol phosphate formation. Half-maximal activation by carbachol is obtained at ~1 μM (7). PLC activity of HEK cells is also stimulated by activation of the endogenously expressed thrombin and purinergic receptors (Fig. 1). However, maximal stimulation compared with that caused by the m3 mAChR was less pronounced. Maximal thrombin-stimulated PLC activation was up to 2-fold, with an EC_{50} value of ~2 units/ml. Activation of purinergic receptors by ATP increased PLC activity by 2–4-fold. Half-maximal and maximal PLC activation was observed at ~0.2 and ~10 μM ATP, respectively. Treatment of the HEK cells with PTX (100 ng/ml, 16 hr) had no effect on inositol phosphate formation stimulated briefly (≤10 min) by either the transfected m3 mAChRs (6–8) or the endogenously expressed thrombin and purinergic receptors (data not shown). As illustrated in Fig. 1, the simultaneous addition of carbachol plus ATP or carbachol plus thrombin, 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. These data thus suggest that common signaling pathways leading to PLC stimulation are shared by the transfected m3 mAChRs and the endogenously expressed thrombin and purinergic receptors and probably involve PTX-insensitive G proteins, most likely, $G_{q/11}$ (6).

Heterologous potentiation of PLC stimulation by carbachol treatment. We recently reported that short term (2-min) treatment of m3 mAChR-expressing HEK cells with carbachol, followed by agonist washout, increases subsequent mAChR-stimulated inositol phosphate formation (8). As exemplified in Fig. 2A, 30 min after the 2-min pretreatment of the cells with 1 mM carbachol, [^{3}H]inositol phosphate production induced by a subsequent challenge with 1 mM carbachol was increased from 13.8 ± 1.1 to 23.2 ± 1.6 × 10^3 cpm/mg of protein (five experiments, $p < 0.001$). As reported previously (8), this sensitization was not accompanied by an increased basal [^{3}H]inositol phosphate production and persisted for ≤~2 hr after agonist removal. We performed studies to characterize the m3 mAChR-mediated PLC sensitization in greater detail, specifically to analyze whether the sensitization is homologous or heterologous. Pretreatment of HEK cells with 1 mM carbachol for 2 min not only potentiated the subsequent stimulation of PLC activity by carbachol but also markedly enhanced stimulation of PLC activity by the endogenously expressed purinergic and thrombin receptors (Fig. 2B). At 30 min after washout of carbachol, stimulation of [^{3}H]inositol phosphate formation induced by ATP (1 mM) was increased from 1.38 ± 0.08 to 3.46 ± 0.14 × 10^3 cpm/mg of protein (five experiments, $p < 0.001$), and subsequent thrombin (4 units/ml)-stimulated [^{3}H]inositol phosphate formation was increased from 1.12 ± 0.05 to 3.34 ± 0.05 × 10^3 cpm/mg of protein (five experiments, $p < 0.001$). Thus, short term carbachol pretreatment caused a heterologous sensitization of receptor-mediated PLC responses. Similar to the potentiation of the m3 mAChR response, the heterologous sensitization of purinergic and thrombin receptor-mediated PLC activation also persisted for at least 90 min after washout of carbachol (data not shown). Analysis of individual inositol phosphate species, specifically of the immediate PLC product Ins(1,4,5)P_3, indicated that carbachol pretreatment potentiated the agonist-induced formation of Ins(1,4,5)P_3 to a similar extent as that of total inositol phosphates. As shown in Fig. 2C, carbachol (1 mM) markedly increased Ins(1,4,5)P_3 mass formation in control cells measured 15 sec after agonist addition. At 30 min after the first carbachol treatment, this increase was increased by 50%. Similar results were obtained when Ins(1,4,5)P_3 mass was determined 30 sec after carbachol rechallenge. Furthermore, qualitatively identical potentiation patterns were observed when the carbachol pretreatment was performed on adherent and nonadherent cells or when production of [^{3}H]Ins(1,4,5)P_3 was determined (instead of Ins(1,4,5)P_3 mass; data not shown).

To study whether activation of other receptors may also induce PLC potentiation, HEK cells were pretreated for 2 min with 1 mM ATP, followed by washout of ATP and subsequent (30 min later) rechallenge with ATP and carbachol. As shown in Fig. 3, treatment of HEK cells with the purinergic receptor agonist ATP (1 mM) did not induce potentiation of the m3 mAChR-mediated PLC activation and also did not increase subsequent ATP-induced PLC stimulation.

The potentiation of receptor-mediated PLC responses was dependent on the carbachol concentration during the pretreatment phase (Fig. 4). Although 10 μM carbachol was rather ineffective, pretreatment of HEK cells with 100 μM carbachol for 2 min increased subsequent m3 mAChR- and purinergic receptor-mediated PLC stimulation approximately half as effectively as pretreatment with 1 mM carba-

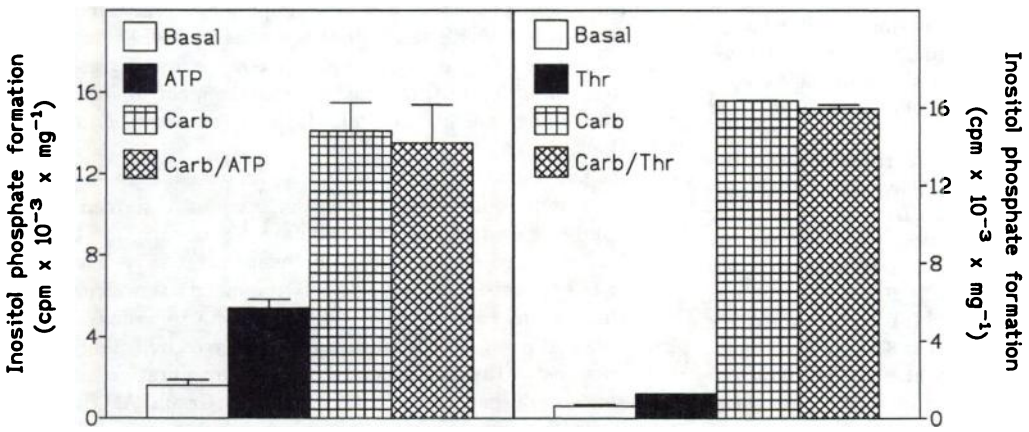

Fig. 1. Activation of PLC by different receptor agonists in m3 mAChR-expressing HEK cells. Formation of [^{3}H]inositol phosphates was determined in *myo*-[^{3}H]inositol-prelabeled HEK cells without (*Basal*) and with (*Carb*) 1 mM carbachol, 1 mM ATP (*left*), 4 units/ml thrombin (*Thr*; *right*), carbachol plus ATP (*Carb/ATP*; *left*), or carbachol plus thrombin (*Carb/Thr*; *right*) as indicated. Data are representative of at least three similar experiments.

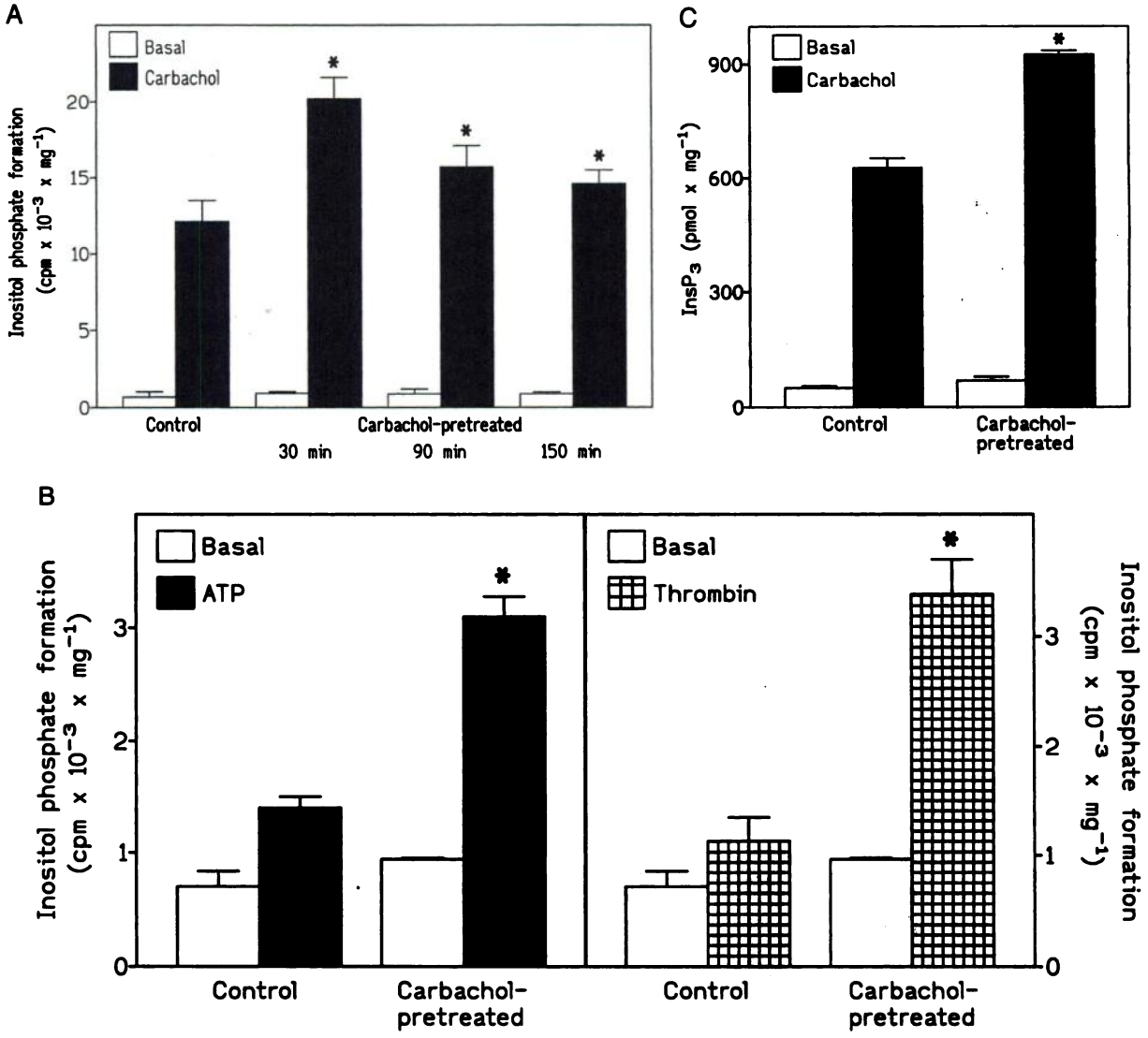

Fig. 2. Long-lasting heterologous sensitization of receptor-mediated PLC responses. A, m3 mAChR-expressing HEK cell monolayers prelabeled with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) and with (*Carbachol-pretreated*) 1 mM carbachol. At the indicated periods of time after washout of carbachol, formation of [^{3}H]inositol phosphates was determined without (*Basal*) and with 1 mM carbachol. B, At 30 min after washout of carbachol, formation of [^{3}H]inositol phosphates was determined without (*Basal*) and with 1 mM ATP or 4 units/ml thrombin. C, At 30 min after washout of carbachol, basal and carbachol (1 mM)-stimulated Ins(1,4,5)P_3 mass formation was determined in unlabeled HEK cells for 15 sec at 37° as described in Experimental Procedures. *, Agonist-stimulated [^{3}H]inositol phosphate or Ins(1,4,5)P_3 mass formation was significantly different ($p < 0.05$) from that of untreated controls.

chol, the highest carbachol concentration examined. Thus, rather high carbachol concentrations are required to induce potentiation of receptor-mediated PLC stimulation. The carbachol-induced potentiation was induced by mAChR activation in that it was prevented by the mAChR antagonist atropine (10 μM) (data not shown). In carbachol-pretreated cells, the carbachol concentration-response curve for PLC stimulation exhibited a pattern quite distinct from that of control cells. Carbachol stimulated [^{3}H]inositol phosphate formation in control cells, with an EC_{50} value of ~1 μM (0.83 μM) and a maximal stimulation at 10 μM (Fig. 5). In carbachol-pretreated cells, the EC_{50} value for carbachol was increased ~4-fold (3.4 μM), and maximal stimulation of PLC activity was barely reached at 1 mM carbachol. Thus, although carbachol pretreatment increased subsequent PLC stimulation at all carbachol concentrations studied, the effect was most pronounced at high agonist concentrations. The Hill coefficients for carbachol stimulation of [^{3}H]inositol phosphate formation in control and carbachol-pretreated cells were calculated to be 0.78 ± 0.33 and 0.38 ± 0.12, respectively.

In the m3 mAChR-expressing HEK cells, PLC activity was also stimulated (2–4-fold) by direct activation of G proteins by AlF_4^- and the stable GTP analog GTPγS. Pretreatment of HEK cells with carbachol also markedly potentiated PLC stimulation by directly activated G proteins. As shown in Fig. 6A, AlF_4^--induced [^{3}H]inositol phosphate formation in intact HEK cells was ~3-fold higher in cells pretreated with 1 mM carbachol than in untreated controls (3.46 ± 0.2 versus 1.3 ± 0.06 × 10^3 cpm/mg of protein, three experiments, $p < 0.001$). Similarly, GTPγS-induced [^{3}H]inositol phosphate formation in digitonin-permeabilized HEK cells pretreated with carbachol was ~2-fold higher than that in untreated control cells (Fig. 6B). Carbachol pretreatment increased the GTPγS (100

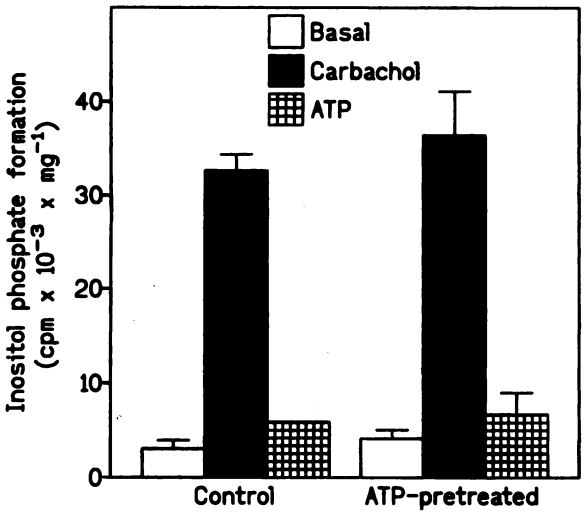

Fig. 3. Lack of PLC sensitization by pretreatment of HEK cells with ATP. m3 mAChR-expressing HEK cell monolayers prelabeled with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) or with (*ATP-pretreated*) 1 mM ATP. At 30 min after washout of ATP, formation of [^{3}H]inositol phosphates was determined without (*Basal*) and with 1 mM carbachol or 1 mM ATP. Data are representative of three similar experiments.

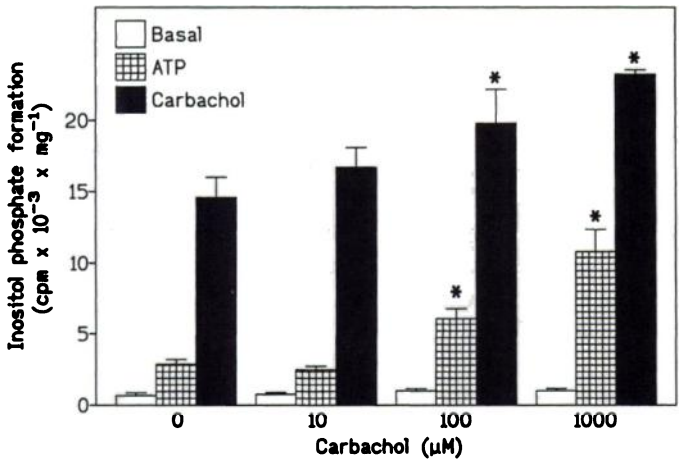

Fig. 4. Agonist concentration dependence of m3 mAChR-induced PLC potentiation. *myo*-[^{3}H]Inositol-prelabeled HEK cells were treated for 2 min with the indicated concentrations of carbachol. At 30 min after washout of carbachol, formation of [^{3}H]inositol phosphates was determined without (*Basal*) and with 1 mM carbachol or 1 mM ATP. Similar data were obtained in three independent experiments. *, Agonist-stimulated [^{3}H]inositol phosphate formation was significantly different ($p < 0.05$) from that of untreated controls.

μM)-stimulated [^{3}H]inositol phosphate formation from 2.54 ± 0.08 to 5.69 ± 0.1 × 10^3 cpm/mg of protein (three 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 cells measured with 40 nM free Ca^{2+}, whereas in intact cells, basal accumulation of [^{3}H]inositol phosphates was not altered (Figs. 2–5).

Role of PtdIns(4,5)P_2 in PLC potentiation. The supply of the PLC substrate PtdIns(4,5)P_2 plays an essential role in receptor signaling to PLC (13, 14). Therefore, we studied whether the carbachol treatment increased the cellular level of PtdIns(4,5)P_2. For this, *myo*-[^{3}H]inositol-prelabeled HEK

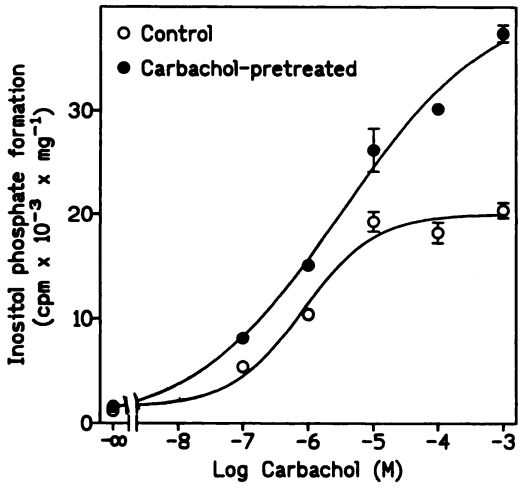

Fig. 5. Alteration in carbachol concentration-response curve in carbachol-pretreated HEK cells. m3 mAChR-expressing HEK cells prelabeled with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) and with (*Carbachol-pretreated*) 1 mM carbachol. At 30 min after washout of carbachol, formation of [^{3}H]inositol phosphates was determined at the indicated carbachol concentrations. Similar data were obtained in three independent experiments.

cells were treated with and without carbachol (1 mM, 2 min), followed by carbachol washout and, 30 min later, extraction and quantification of the labeled phosphoinositide species (i.e., at the exact time point used for measurement of inositol phosphate formation). Short term carbachol pretreatment had no effect on the level of [^{3}H]PtdIns compared with untreated controls, whereas the cellular level of [^{3}H]PtdIns4P was reduced by 40–50% (Fig. 7A). Most important, the carbachol pretreatment increased significantly ($p < 0.01$; by ~50%) the level of [^{3}H]PtdIns(4,5)P_2 in HEK cells. A similar, ~50% increase was observed when PtdIns(4,5)P_2 mass was determined in control and carbachol-pretreated HEK cells (Fig. 7B).

If the increase in cellular PtdIns(4,5)P_2 level caused by carbachol pretreatment was responsible for the observed PLC sensitization, inositol phosphate formation should not be enhanced when PLC activity is measured with exogenous PtdIns(4,5)P_2 as enzyme substrate. As demonstrated in Fig. 8, this was indeed the case. Basal PLC activity measured with [^{3}H]PtdIns(4,5)P_2 (50 μM) as enzyme substrate was not different in HEK cell lysates obtained from either untreated controls or carbachol-pretreated HEK cells. Most important, activation of PLC in cell lysates by directly activated G proteins, using exogenous PtdIns(4,5)P_2 as enzyme substrate, also was not affected by carbachol pretreatment. In either cell lysate, GTPγS and AlF_4^- stimulated PLC activity was by a similar 3- and 4-fold, respectively (Fig. 8).

Involvement of G_i proteins in PLC potentiation. In addition to activating $G_{q/11}$ proteins, m3 mAChRs can couple to G_i-type (G_{i1}/G_{i3}) G proteins in HEK cells (6). To study whether this m3 mAChR/G_i coupling is involved in PLC potentiation, the m3 mAChR-expressing HEK cells were pretreated with PTX (100 ng/ml, 16 hr) before the short term (2-min) treatment with 1 mM carbachol. In a confirmation of previous results (6, 7), PTX treatment had no significant effect on the carbachol-stimulated [^{3}H]inositol phosphate formation in control cells (Fig. 9A). However, the carbachol-induced sensitization of PLC stimulation was completely pre-

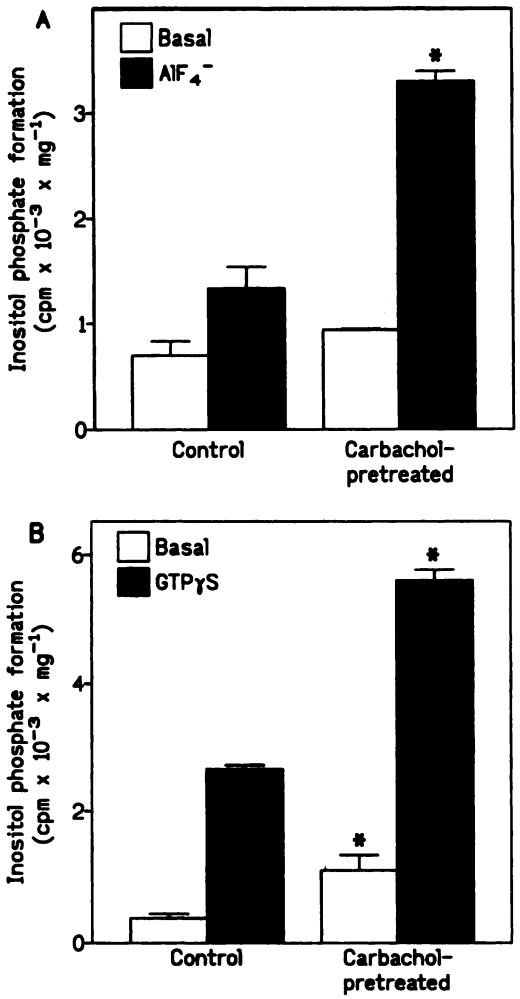

Fig. 6. Sensitization of G protein-mediated PLC stimulation. m3 mAChR-expressing HEK cells prelabeled with *myo*-[^{3}H]inositol were pretreated for 2 min without (*Control*) and with (*Carbachol-pretreated*) 1 mM carbachol. A, At 30 min after washout of carbachol, formation of [^{3}H]inositol phosphates was determined in intact cells without (*Basal*) and with AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$). B, At 30 min after washout of carbachol, formation of [^{3}H]inositol phosphates was determined in digitonin-permeabilized adherent HEK cells without (*Basal*) and with GTPγS (100 μM) as described in Experimental Procedures. *, [^{3}H]Inositol phosphate formation was significantly different ($p < 0.05$) from that of untreated controls.

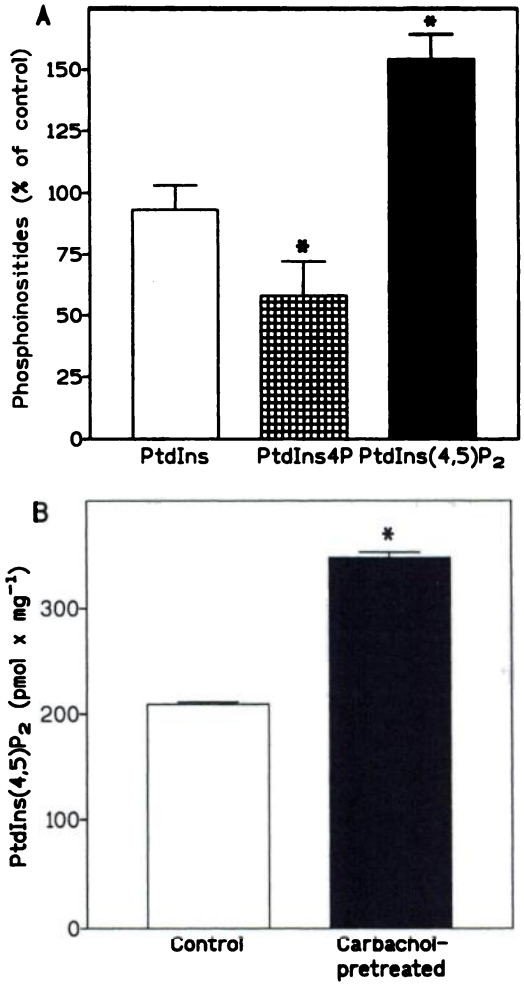

Fig. 7. Effects of carbachol pretreatment on the level of phosphoinositides in HEK cells. The level of phosphoinositides was quantified in m3 mAChR-expressing HEK cells pretreated for 2 min with and without 1 mM carbachol. A, At 30 min after washout of carbachol, the levels of [^{3}H]PtdIns, [^{3}H]PtdIns4P, and [^{3}H]PtdIns(4,5)P_2 were determined in *myo*-[^{3}H]inositol-prelabeled HEK cells as described in Experimental Procedures and are given as a percentage of control (i.e., in cells not treated with carbachol). In untreated control cells, the radioactivity associated with [^{3}H]PtdIns, [^{3}H]PtdIns4P, and [^{3}H]PtdIns(4,5)P_2 was 24.9 ± 2.6, 2.76 ± 0.19, and 4.4 ± 1.0 × 10^3 cpm/mg of protein, respectively. Data are from three separate experiments. B, At 30 min after washout of carbachol, PtdIns(4,5)P_2 mass was determined in unlabeled HEK cells as described in Experimental Procedures. Data are from two separate experiments. *, Significantly different ($p < 0.01$) from untreated controls.

vented by prior PTX treatment. In PTX-treated HEK cells, which were also pretreated with and without carbachol (1 mM, 2 min), carbachol (1 mM) increased [^{3}H]inositol phosphate formation by 10.8 ± 0.3 and 10.3 ± 0.3 × 10^3 cpm/mg of protein, respectively (five experiments). Similar results were obtained with ATP used as a second stimulus in control and PTX-treated cells that were also pretreated with and without carbachol (data not shown). Finally, the effect of PTX treatment on carbachol-induced alterations in phosphoinositide levels was determined. As shown in Fig. 9B, PTX treatment did not affect the carbachol-induced reduction in [^{3}H]PtdIns4P level; the reduction was ~50%, which was similar to non-PTX-treated cells. However, PTX treatment reduced, although not completely, the carbachol-induced increase in the level of [^{3}H]PtdIns(4,5)P_2 from a 50% increase above control level in nonintoxicated cells (see Fig. 7A) to an only 25% increase in PTX-pretreated cells.

Discussion

Based on the recent observation that short term activation of m3 mAChRs stably expressed in HEK cells can lead to a long-lasting sensitization of mAChR-stimulated inositol phosphate production (8), the aim of the current study was to characterize this long term PLC potentiation in greater detail and analyze potential underlying mechanisms. We demonstrate here that the 2-min pretreatment of m3 mAChR-expressing HEK cells leads not only to a potentiation of subsequent mAChR-stimulated PLC activity but also to a pronounced potentiation of PLC stimulation by the endogenously expressed thrombin and purinergic receptors. All of these receptors seem to activate PLC via common signaling pathways that involve PTX-insensitive G proteins, most

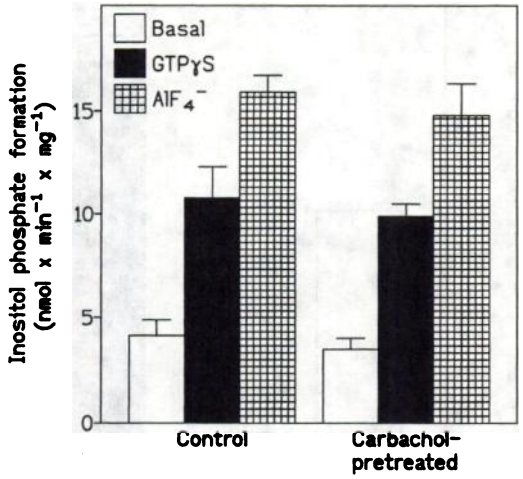

Fig. 8. Lack of PLC sensitization measured with exogenous substrate. m3 mAChR-expressing unlabeled HEK cells were pretreated for 2 min without (*Control*) and with (*Carbachol-pretreated*) 1 mM carbachol. At 30 min after washout of carbachol, formation of [^{3}H]inositol phosphates from exogenous [^{3}H]PtdIns(4,5)P_2 was determined in HEK lysates in the absence (*Basal*) and presence of GTPγS (100 μM) or AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$) as described in Experimental Procedures. Data are representative of at least four similar experiments.

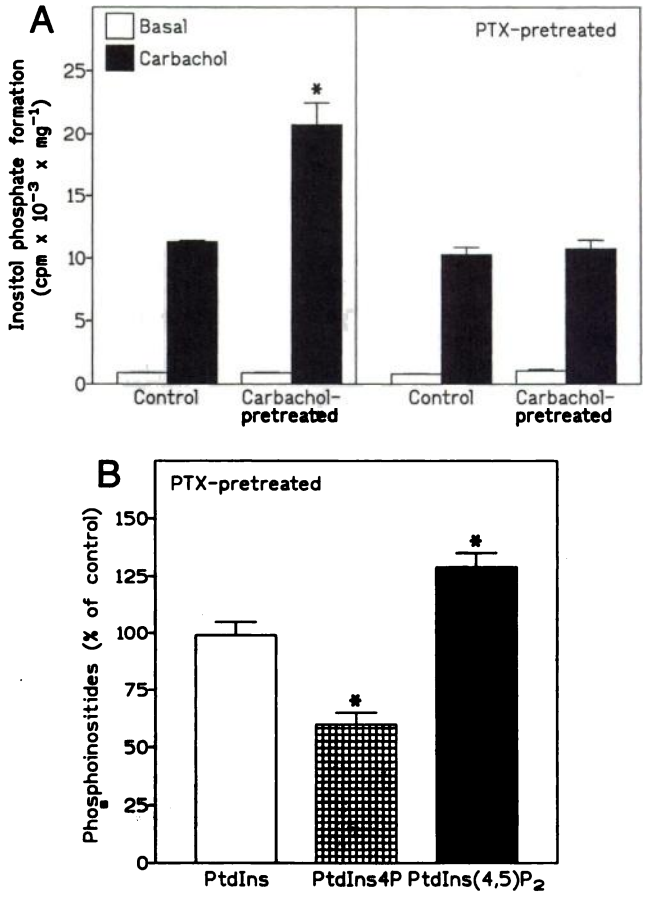

Fig. 9. Influence of PTX treatment on PLC sensitization and elevation in PtdIns(4,5)P_2 level. A, m3 mAChR-expressing and *myo*-[^{3}H]inositol-prelabeled HEK cells were pretreated for 16 hr without (*right*) and with (*left*) 100 ng/ml PTX and then for 2 min without (*Control*) and with 1 mM carbachol (*Carbachol-pretreated*) as described in Experimental Procedures. At 30 min after washout of carbachol, [^{3}H]inositol phosphate formation was determined without (*Basal*) and with 1 mM carbachol. Data are representative of five similar experiments. B, At 30 min after washout of carbachol, the levels of [^{3}H]PtdIns, [^{3}H]PtdIns4P, and [^{3}H]PtdIns(4,5)P_2 were determined in m3 mAChR-expressing HEK cells pretreated for 16 hr with 100 ng/ml PTX and treated subsequently for 2 min without and with 1 mM carbachol and are given as a percentage of control (i.e., in cells not treated with carbachol). In control cells, the radioactivity associated with [^{3}H]PtdIns, [^{3}H]PtdIns4P, and [^{3}H]PtdIns(4,5)P_2 was 30.6 ± 0.4, 0.70 ± 0.02, and 1.44 ± 0.40 × 10^3 cpm/mg of protein, respectively. Data are from three separate experiments. *, Significantly different ($p < 0.05$) from untreated controls.

likely, $G_{q/11}$, as demonstrated for the m3 mAChR (6). The sensitization of receptor-stimulated inositol phosphate formation persisted for at least 90 min after washout of carbachol and was also detectable on the level of the immediate PLC product Ins(1,4,5)P_3, measured as accumulation of [^{3}H]Ins(1,4,5)P_3 (data not shown) or Ins(1,4,5)P_3 mass. Thus, the carbachol-induced potentiation of PLC stimulation was heterologous at the receptor level. Therefore, we studied whether G protein-mediated PLC stimulation is also potentiated by carbachol treatment. Using AlF_4^- and the stable GTP analog GTPγS to activate G proteins in intact and permeabilized HEK cells, respectively, it was demonstrated that the carbachol pretreatment also enhanced PLC stimulation caused by direct G protein activation. These data suggested that the sensitization of the PLC response was not due to a facilitated receptor coupling to PLC-stimulating G proteins but rather that a mechanism downstream of receptors and G proteins is responsible for the long term potentiation.

Potentiation of G protein-coupled receptor PLC signaling has been described previously. However, in most cases, this potentiation required long term (24-hr) cell treatment [e.g., of HL-60 cells with retinoic acid (15) or of human keratinocytes with 1,25-dihydroxy vitamin D_3 (16)] and was apparently caused by overexpression of a specific G protein subunit (γ_2) involved in receptor signaling (15) or a PLC-β isozyme (PLC-β_1) (16). Furthermore, down-regulation of protein kinase C subtypes α and δ in rat astrocytes by long term treatment (6–24 hr) with the phorbol ester phorbol-12-myristate-13-acetate has been shown to induce potentiation of bradykinin-induced PLC stimulation (17). This potentiation was attributed to new synthesis of bradykinin receptors and G proteins (17). It is unlikely that such mechanisms underlie the carbachol-induced potentiation of PLC stimulation in HEK cells. First, the agonist treatment was very short (2 min), and the potentiation was observed 30 min after washout of carbachol. Second, using exogenous PtdIns(4,5)P_2 to measure PLC activity in HEK cell lysates, no differences in basal enzyme activity or PLC activities stimulated by either directly activated G proteins (AlF_4^-, GTPγS) or Ca^{2+} (data now shown) were noted regardless of whether the cells were pretreated with carbachol. This finding prompted us to study whether the short term carbachol treatment may have increased the level of the PLC substrate PtdIns(4,5)P_2, thereby leading to an increased production of inositol phosphates by receptor agonists and directly activated G proteins. Analysis of the cellular phosphoinositides 30 min after washout of carbachol (i.e., at the time point when inositol phosphate formation was studied) indicated that the 2-min carbachol pretreatment had increased the total cellular level of PtdIns(4,5)P_2, measured as [^{3}H]PtdIns(4,5)P_2 or PtdIns(4,5)P_2 mass, by ~50%, whereas that of PtdIns was unchanged, and that of PtdIns4P was reduced by ~50%. These data suggest that carbachol, in

addition to inducing PLC stimulation, may increase PtdIns4P 5-kinase activity in HEK cells. Indeed, it has been reported that G protein-coupled receptors can cause activation of PtdIns4P 5-kinase activity (18, 19). Furthermore, as demonstrated in rat pancreatic acinar cells, the mAChR-stimulated synthesis of PtdIns(4,5)P_2 by PtdIns4P 5-kinase was retained for 3 hr after agonist washout, suggesting a stable covalent modification of the enzyme (19). Activation of PtdIns4P 5-kinase may also account for the rather rapid resynthesis of PtdIns(4,5)P_2 after the initial decrease in its level, despite the continuous presence of the agonist [e.g., as described in m1 mAChR-expressing Chinese hamster ovary cells (20)]. The increase in PtdIns(4,5)P_2 level, however, apparently only in part accounts for the observed PLC potentiation in HEK cells (see below). In rat basophilic leukemia RBL-2H3 cells, priming of PLC responses of various chemoattractant receptors by thrombin has been reported that was not associated with significant alterations in total PtdIns(4,5)P_2 level (21).

We recently reported that the m3 mAChR couples to and activates not only $G_{q/11}$ but also G_i-type (G_{i1}/G_{i3}) G proteins in HEK cells (6). However, no functional response could be ascribed to this G_i coupling, and compared with the coupling to $G_{q/11}$, activation of G_i proteins by the m3 AChR was observed only at rather high agonist (carbachol) concentrations (≥10 μM) (6). The data presented in the current study strongly suggest that the carbachol-induced sensitization of PLC-catalyzed inositol phosphate formation is caused by activation of G_i proteins. This is supported by the following observations. First, PTX treatment completely prevented the carbachol-induced potentiation of PLC stimulation, indicating that carbachol-activated G_i proteins induce a cellular process that is responsible for the subsequent potentiation of PLC stimulation and/or that in carbachol-pretreated cells the m3 mAChR activates PLC not only via $G_{q/11}$ proteins but also via G_i proteins. Various data suggest that either mechanism is probably involved in potentiation of PLC stimulation. First, PTX treatment, although completely preventing the potentiation of m3 mAChR-stimulated inositol phosphate formation, inhibited only partially the increase in PtdIns(4,5)P_2 level induced by carbachol treatment (50%). Second, in carbachol-pretreated HEK cells, the carbachol concentration-response curve for PLC stimulation was significantly different from that in control cells. In control cells, carbachol increased PLC activity with a concentration-response curve very similar to that observed for carbachol-induced activation of $G_{q/11}$ proteins (i.e., with an EC_{50} value of ~1 μM and a maximal activation at 10 μM) (6). On the other hand, in carbachol-pretreated cells, the PLC activation concentration-response curve exhibited a Hill coefficient of ~0.4, suggesting mediation by two distinct processes. The potentiation of PLC stimulation, although observed at all carbachol concentrations studied, was most obvious at high carbachol concentrations (>10 μM), which is very similar to those required for activation of G_i proteins in HEK cell membranes (6). Thus, although other mechanisms cannot be excluded, we suggest that acute stimulation of PLC, most likely a PLC-β isoenzyme or isoenzymes, by the transfected m3 mAChRs and the endogenously expressed thrombin and purinergic (P_2-type) receptors is mediated by $G_{q/11}$ (Fig. 10). On the other hand, the m3 mAChR-induced potentiation of PLC stimulation seems to be caused by activation of G_i proteins,

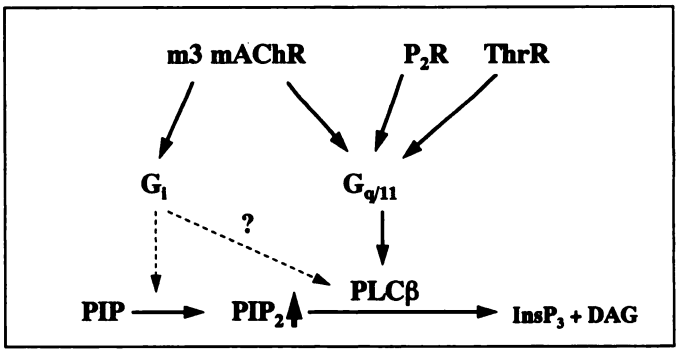

Fig. 10. Participation of $G_{q/11}$ and G_i in receptor-induced activation and sensitization, respectively, of PLC in HEK cells. *P_2R*, P_2 purinergic receptor; *ThrR*, thrombin receptor; *DAG*, diacylglycerol; *PIP*, phosphatidylinositol monophosphate; *PIP_2*, phosphatidylinositol bisphosphate; *$InsP_3$*, Ins(1,4,5)P_3. (For further details, see the text.)

mediating at least partially the agonist-induced increase in PtdIns(4,5)P_2 level and probably also directly involved in m3 mAChR-mediated PLC stimulation in carbachol-pretreated cells.

Previous reports have described PTX-sensitive potentiation of PLC stimulation by simultaneous activation of a receptor that is coupled via $G_{q/11}$ to PLC and a receptor that is coupled to PTX-sensitive G proteins and does not alone induce PLC activation. For example, in FRTL-5 thyroid cells, phenylisopropyladenosine, an A_1 receptor agonist, has been reported to potentiate the P_2 purinergic receptor-induced PLC stimulation in a PTX-sensitive manner (22). Similarly, in mouse striatal astrocytes, somatostatin has been demonstrated to potentiate the α_1-adrenergic receptor-induced PLC stimulation in a PTX-sensitive manner (23). However, the underlying mechanisms were either not further analyzed (22) or reported to involve arachidonic acid and glutamate release (23). Most important, in neither case was it studied whether the PTX-sensitive potentiation of the PLC response was long lasting (i.e., observed after removal of the sensitizing agonist). There are two reports in the literature demonstrating a long-lasting G protein-coupled receptor-induced sensitization of PLC stimulation in intact tissues (24, 25). Preincubation of rat anterior pituitary glands with luteinizing hormone-releasing hormone for 1 hr, followed by agonist washout, increased subsequent Ins(1,4,5)P_3 production by the same hormone by ~2-fold (24). Furthermore, pretreatment of rats by intracerebroventricular injection of vasopressin caused a sensitization of subsequently measured (24 hr later) vasopressin-induced inositol phosphate formation in septal slices (25). These results, which were not analyzed further, were interpreted as facilitated receptor coupling to PLC (24, 25). Thus, although we failed to observe an ATP-induced PLC potentiation in HEK cells under the conditions studied, these data suggest that receptor-induced long term potentiation of PLC stimulation may not be restricted to a cellular system with transfected and overexpressed receptors (i.e., m3 mAChR-expressing HEK cells) but may also be found with endogenously expressed G protein-coupled receptors.

In conclusion, the data presented here indicate that short term agonist treatment of m3 mAChR-expressing HEK cells induces a long-lasting heterologous potentiation of receptor signaling to PLC. The potentiation apparently involves G_i

proteins and an enhanced synthesis of the PLC substrate PtdIns(4,5)P_2. Studies are in progress to analyze the mechanisms involved in receptor-induced PtdIns(4,5)P_2 synthesis and concomitant potentiation of PLC stimulation. Because Ins(1,4,5)P_3 and diacylglycerol, the products of PLC-catalyzed hydrolysis of PtdIns(4,5)P_2, can apparently control a variety of early and late cellular processes, the receptor-induced long term potentiation of PLC product formation that we reported may constitute a novel mechanism of long term cellular plasticity.

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