**Pasteurella multocida** toxin activates G\(\beta\)\(\gamma\) dimers of heterotrimeric G proteins

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**A B S T R A C T**

The mitogenic Pasteurella multocida toxin (PMT) is a major virulence factor of *P. multocida*, which causes Pasteurellosis in man and animals. The toxin activates the small GTPase RhoA, the MAP kinase ERK and STAT proteins via the stimulation of members of two G protein families, G\(\alpha\)\(q\) and G\(\alpha\)\(12/13\) [8]. PMT action also results in an increase in inositol phosphates, which is due to the stimulation of PLC\(\gamma\) via G\(\alpha\)\(q\). Recent studies indicate that PMT additionally activates G\(\alpha\)\(i\) to inhibit adenylyl cyclase. Here we show that PMT acts not only via G\(\alpha\)\(q\) but also through G\(\beta\)\(\gamma\) signaling. Activation of G\(\beta\)\(\gamma\) by PMT causes stimulation of phosphoinositide 3-kinase (PI3K)\(\gamma\) and formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) as indicated by the recruitment of a PIP3-binding pleckstrin homology (PH) domain-containing protein to the plasma membrane. Moreover, it is demonstrated that G\(\beta\)\(\gamma\) is necessary for PMT-induced signaling via G\(\alpha\)\(q\). Mutants of G\(\alpha\)\(q\) incapable of binding or releasing G\(\beta\)\(\gamma\) are not activated by PMT. Similarly, sequestration of G\(\beta\)\(\gamma\) inhibits PMT-induced Go-signaling.

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**1. Introduction**

The 146 kDa protein toxin (PMT) from *Pasteurella multocida* is one major virulence factor of the pathogen [1]. Whereas the role of PMT in human *P. multocida*-induced diseases has not been elucidated, the toxin has been shown to cause various diseases of wild-life and domestic animals, including the atrophic rhinitis of pigs, which is characterized by major destruction of the nose turbinate [2,3]. In cell culture of fibroblasts, PMT is one of the most potent mitogens known [4]. PMT stimulates several signaling pathways. The toxin activates the small GTPase Rho, the MAP kinase ERK and STAT proteins [5–7]. These effects are mainly caused by activation of members of two heterotrimeric G protein families, G\(\alpha\)\(q\) and G\(\alpha\)\(12/13\) [8]. Quite early it was recognized that PMT is a strong stimulator of G\(\alpha\)\(i\), resulting in phospholipase C\(\gamma\) activation [4,9]. Notably, G\(\alpha\)\(12/13\), which couples to same receptors as G\(\alpha\)\(q\), and also activates phospholipase C, is not stimulated by the toxin [8,10]. Stimulation of G proteins by PMT is independent of coupling to receptors and virtually permanent [11]. More recently, we reported that also G\(\alpha\)\(i\) is potently activated by PMT [12]. However, the precise molecular mechanism underlying the PMT-induced activation of G proteins is not known. Recently, the crystal structure of a large fragment of PMT, including the biologically active domain, has been solved, suggesting that PMT acts as a cysteine protease [13].

Activation of G proteins by G-protein-coupled receptors (GPCR) causes signaling via G\(\alpha\)\(q\) and/or G\(\beta\)\(\gamma\) subunits. However, it has not been shown whether GPCR-independent activation of G proteins by PMT also results in activation of G\(\beta\)\(\gamma\) and subsequent signaling. Here we report that PMT releases G\(\beta\)\(\gamma\) from heterotrimeric G proteins, thereby activating the PI3K pathway. Moreover, we demonstrate that G\(\beta\)\(\gamma\) is necessary for PMT-induced activation of G\(\alpha\)\(q\).

**2. Experimental procedures**

2.1. Materials

Myo-[2-3H]inositol was obtained from Perkin Elmer. All other reagents were of analytical grade and purchased from commercial sources.

2.2. Construction of expression plasmids

Construction of PH domain of general receptor for phosphoinositides-1 (Grp1PH) in pEGFP-C1 [aa 241-386] and p101, p110\(\gamma\), G\(\beta\)\(1\), G\(\beta\)\(2\).
flag-NF1-333 (NF1), CFP-GRK2-CT [aa 570-689 of G-protein-coupled-receptor kinase 1] (GRK is also known as βARK2), phosducin (Pd) and the human formyl-methionyl-leucyl-phenylalanine (fMLP) receptor in pcDNA3 has been described elsewhere [14–17].

2.3. Cell culture, transfection, virus production and transduction

HEK293 cells (from the German Resource Center for Biological Material) were grown at 37 °C with 5% CO2 in DMEM with Earle’s Salts (Gibco BRL) supplemented with 10% FCS (Gibco BRL), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gentaxxon). HEK293 cells stably expressing the muscarinic acetylcholine receptor M3 [18] were cultured like HEK293 wild-type cells, but in the presence of G418 (0.5 mg/ml). For confocal analysis, cells were seeded on glass cover slips two days prior to transfection. All transfections were done with a calcium phosphate-based transfection method. HEK293 cells were transfected with 0.2 μg of plasmid encoding a formyl-methionyl-leucyl-phenylalanine (fMLP) receptor, 0.4 μg of each of the plasmids encoding the PI3K subunits and the fluorescent PH domain, 1.0 μg of each of the plasmids encoding Gq11, Gq21, and 1.1 μg each of the plasmids encoding NF1 and GRK2-CT. The total amount of transfected cDNA was always kept constant (2.5 μg/well) by the addition of empty expression vector. All experiments were performed at least 24 h after transfection in serum-starved cells.

Mouse embryonic fibroblasts (MEF) derived from Gαq/Gα11-deficient or wild-type (wt) mice were cultured as described before [19]. The retroviral vector was produced as described before [20]. In brief, HEK-293T cells were co-transfected with pMD-G, pMD-g/p and the retroviral transfer vector. The calcium phosphate method was used. The supernatant was collected after 3 days and centrifuged to spin down cellular debris. The virus-containing medium was filtered. Cells were infected in the presence of Polybrene. The expression was monitored by Western-blot analysis. Additional transfection of mouse embryonic fibroblasts.

2.4. Confocal laser scanning microscopy

Cell imaging was established and performed as described previously with modifications outlined below [14,21,22]. Glass coverslips were mounted on a custom-made chamber and covered with 200 μl of HEPES-buffered solution (HBS), containing 138 mM NaCl, 6 mM KCl, 5.5 mM glucose, and 10 mM HEPES, pH 7.5. For confocal imaging, an inverted confocal laser scanning microscope with a Plan-Apochromat 63/1.4 objective (model LSM 510-Meta; Carl Zeiss Microlimaging Inc.) was used. To maintain a constant temperature of 37 °C for live cell imaging, the confocal microscope device was equipped with a ZEISS Incubator XL. GFP and Alexa633 were excited at 488 nm and 633 nm, and the fluorescence emission was detected through a 505–530 nm band pass filter and a 650 nm long pass filter, respectively. The pinholes were adjusted to yield optical sections of 0.5–1.0 μm. Cells were pre-treated with pertussis toxin (PTX; 200 ng/ml, over night) or YM-254890 (1 μM, for 15 min prior to GPCR stimulation or 5 h when stimulated with PMT). Stimulation of the cells with the toxin was performed for 5 h at a concentration of 1 nM PMT. In order to stimulate the GPCRs, the cells were treated with 1 μM fMLP (Merck) or 100 μM carbacol (Sigma) for 2–5 min.

2.5. Semi-quantitative analysis of GFP-Grp1PH translocation

The GFP-labeled PH domain of the general receptor for phosphoinositides-1 (GFP-Grp1PH) binds specifically to PIP1. Therefore the subcellular distribution of GFP-Grp1PH reflects the activity of PI3Ks, i.e. enhancement of membrane-associated fluorescence was taken as an indicator of PI3K activation.

For semi-quantitative analysis of PI3K activation in PMT pre-treated cells the ratio of the fluorescence intensities of GFP-Grp1PH at the cell surface and the cell interior of the confocal plane were calculated (Fsurf/Fcyto) and compared to control cells. Fsurf represents the mean intensity of fluorescence per μm² associated with the plasma membrane compartment whereas Fcyto represents the mean intensity of the intracellular fluorescence per μm². For statistical analysis of PI3K activity means and standard deviations were calculated from three independent experiments based on different transfections analyzing six cells each.

The semi-quantitative evaluation of the kinetics of receptor-induced PI3K activation was not based on the increasing fluorescence intensity at the membrane since values quickly reached saturation. Instead, we measured the simultaneous decrease of the cytosolic fluorescence intensity. This approach is based on the assumption that the disappearance of GFP-Grp1PH-dependent fluorescence from the cytosol is almost completely due to its translocation to the plasma membrane except for the bleaching effect which is covered by control experiments. Accordingly, shortly before and after application of the stimulus successive pictures were taken (every 10 s for 3–4 min).

The relative amount of GFP-Grp1PH domain translocated at the time (t) is given by this equation: relative PI3K activity=([Fcyto(0)–Fcyto(t)]/[Fcyto(0)×100].

2.6. Site-directed mutagenesis

Mutations were introduced by site-directed mutagenesis, using Gαq cloned into pLNCX2 as template [8] and the respective oligonucleotides, using the Quick-Change kit, according to the manufacturer’s instructions. From the two complementary primers used for each mutation, only one is listed: Gαq5′-ATC AAC GAC GAG -3′; GRK2-CTR587Q, 5′-GTC GAT GTA GGG GCC CAA ACG TCA GAG A-3′; Gαq5′-ATC AAC GAC GAG GCC GGG CAC GTG GGC-3′; GRK2-CT-453Q, 5′-CCT GTT CCC TAA CCA CCT CGA GTG GGG GCC C-3′. All mutations were confirmed by DNA sequencing with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer).

2.7. Assay of SRF activation

HEK293-M3 cells were transfected with the pSRE.L-luciferase reporter plasmid and the pRL.TK control reporter vector. pSRE.L encodes for firefly (Photinus pyralis) luciferase; the expression is induced by activation of SRF. pRL.TK encodes for Renilla (Renilla reniformis) luciferase, which is expressed constitutively. The plasmids pSRE.L and pRL.TK were kind gifts of Dr. R. Treisman (Transcription Laboratory, Imperial Cancer Research Fund Laboratories, London, England). Cells were transfected using the jetPEI-transfection Kit from Biomol. Cultures were serum-starved for 24 h before stimulation with PMT for additional 20 h and lysed with passive lysis buffer (Promega). Luciferase activities were determined with the Dual-Luciferase reporter assay system (Promega) in accordance to the manufacturer’s instructions. The activity of the experimental reporter was normalized against the activity of the control vector.

2.8. Analysis of total inositol phosphates

Gαq11-deficient MEF transfected with retroviral constructs were grown in 24-well plates for 2 to 3 days. Then, cultures were labeled.
with 2 μCi/ml of [2-3H]inositol in inositol- and serum-free medium over night. PMT was added at the indicated concentration and incubated for the indicated time. LiCl (20 mM) was added 60 min before assay was stopped to allow accumulation of inositol phosphates. Thereafter, the medium was replaced by 750 μl of ice-cold formic acid (10 mM, pH 3). After 30 min, the extract was neutralized with 3 ml of NH₃ (5 mM, pH 8–9). Analysis of total inositol phosphates was done by anion exchange chromatography as described before [24].

2.9. Expression of recombinant proteins

Recombinant PMTwt and the inactive mutant PMT[C1165S] were expressed and purified as described before [24].
2.10. Immunoblot analysis

Cells were extracted at 4 °C with RIPA buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1% Nonidet P-40, 0.5% (w/v) deoxylcholate, 0.1% (w/v) SDS, and complete protease inhibitors, Roche Applied Science) and analyzed by Western-blotting after SDS-polyacrylamide gel electrophoresis. Anti Goα₁1-1, anti flag-M2 and anti GRK2-CT antibodies were purchased from Santa Cruz Biotechnology, Sigma and Epitomics, respectively. Binding of the second horseradish peroxidase-coupled antibody was detected with enhanced chemiluminescent detection reagent (100 mM Tris–HCl, pH 8.0, 1 mM luminol (Fluka), 0.2 mM p-coumaric acid, and 3 mM H₂O₂), and the imaging system LAS-3000 (Fujifilm).

3. Results

3.1. Activation of PI3Kγ by PMT

It has been shown that PMT activates Go in a receptor-independent manner [11]. This results in activation of PLC-β by Goα [9,11]. To study whether PMT induces not only activation of Goα- but also of Goβγ-dependent pathways, we transfected HEK293 cells stably expressing the Goγ-coupled M₃ receptor (Fig. S2B) with plasmids encoding a heterodimeric PI3Kγ, a prototypical Goβγ effector, which is not endogenously expressed in HEK cells [14,25,26]. Activation of PI3Kγ was assessed by translocation of the PIP₃-sensor GFP-Grp1PH from the cytosol to the membrane (Fig. 1). In the absence of PI3Kγ PMT (1 nM) had no effect on redistribution of GFP-Grp1PH indicating that the
endogenously expressed receptor-tyrosine-kinase-regulated PI3Ks were insensitive [14]. Vice versa the sole over-expression of PI3Kγ did not induce redistribution of the PIP₃ sensor. However, PMT-treatment of HEK293 cells over-expressing PI3Kγ strongly stimulated the translocation of the GFP-Grp1ₚₜ to membranes. This effect was not observed with the inactive PMT mutant (PMT<sup>C1165S</sup>), which was applied at 10 nM.

PI3Kγ is stimulated by Gβγ dimers and Ras proteins but not by Gα subunits [25,27]. The C-terminal part of GRK-2 (GRK2-CT) binds Gβγ and can be used to sequester Gβγ [28,29]. Over-expression of the Gβγ-scavenger GRK2-CT blunted activation of PI3Kγ (Fig. 1). In contrast, inactivation of Ras by the GAP domain of NF1 did not significantly affect PI3Kγ activity (Fig. 1, lower panel). Of note functional expression of NF1 was confirmed by immunoblot analysis.

Fig. 3. PMT leads to Gβγ-dependent activation of the PI3Kγ in HEK293 cells. A. In each set of parallel experiments HEK293 cells were transfected with the identical mixture of plasmids as indicated in the figure. Treatment of cells was without (b) and with wild-type PMT (a, c, f and g), inactive PMT mutant (d, 10 nM PMT<sup>C1165S</sup>) and wild-type PMT plus compound YM-254890 (e, 1 nM, PMT<sup>wt</sup> + 1 µM YM-254890) for 5 h. In f, the effect of the Gβγ-scavenger GRK2-CT is shown; g shows the effect of the Ras-GAP NF1. B. Histogram showing the quantification of the membrane translocation of GFP-Grp1ₚₜ from (A). In addition, a control with overexpression of Gβγ (Gβγ) is shown. The membrane translocation of GFP-Grp1ₚₜ is evaluated as the ratio: F<sub>membrane</sub>/F<sub>cytoplasm</sub> (mean intensity of fluorescence per µm² associated with the membrane/mean intensity of intracellular fluorescence per µm²; for details see “Experimental Procedures” section). Data depicted represent the mean ± SD from 3 independent sets of experiments analyzing 18 cells. Statistical significance was assessed using a paired Student’s t-test, with *p < 0.05 and **p < 0.01.
and a Ras activity assay (Fig. S2A). These findings indicate that PMT signals not only via \( \text{Gq} \) but also via \( \text{G}\gamma \).

### 3.2. Involvement of different G proteins in PI3K activation by PMT

Recently, we reported that also \( \text{G}\gamma \) is potently activated by PMT [12]. To test whether \( \text{G}\gamma \) is the only source of PMT-released \( \text{G}\gamma \) dimers, we used the \( \text{Gq} \)-inhibitor YM-254890, which prevents activation of \( \text{Gq} \) not only by receptors but also by PMT [30,31]. For control, it was initially confirmed that YM-254890 inhibited \( \text{G}\gamma \) signaling from GPCR-activated \( \text{G}\alpha\beta\gamma \) complex (Fig. 2). To this end, the effects of the muscarinic agonist carbachol were studied on GFP-Grp1PH translocation-dependent luciferase activation was measured in the presence and contrast, after transfection of \( \text{G}\alpha q \)-inhibitor YM-254890 (Fig. 1), suggesting the involvement of additional \( \text{G}\gamma \) protein isoforms beside \( \text{Gq} \) such as \( \text{Gi} \). In the case of PMT we could not use PTX to identify \( \text{G}\gamma \) as an additional source for \( \text{G}\gamma \), because it is known that PMT activates \( \text{G}\gamma \) even after ADP-ribosylation by PTX [12].

Similar results were obtained in HEK293 cells without stable expression of the \( \text{M}\gamma \) receptor (Fig. 3). For semi-quantitative comparison of PMT-induced PI3K activity, \( \text{G}\gamma \)-dependent luciferase activity was transfected in HEK cells which elicits maximal stimulation of the enzyme. Only a slightly higher activity of PI3K was observed in cells over-expressing \( \text{G}\gamma \) compared to PMT-treated cells (Fig. 3B).

#### 3.3. \( \text{G}\gamma \) is essential for activation of G proteins by PMT

As our studies suggested that \( \text{G}\gamma \) subunits play a crucial role in PI3K activation by PMT, we investigated the effects of the toxin on \( \text{G}\gamma \) release (activation) in more detail. For this purpose we employed mutant \( \text{G}\gamma \) proteins, which are known to be defective in \( \text{G}\gamma \)-cycling. The \( \text{G}\gamma \) double mutant I25A/E26A has lost its ability to bind the \( \text{G}\gamma \)-dimers [32,33]. In contrast, the G208A mutant of \( \text{G}\gamma \) is known to permanently bind \( \text{G}\gamma \) and is not able to release \( \text{G}\gamma \) upon \( \text{G}\alpha \) interaction with agonist-bound receptors [34–36]. The mutant proteins were expressed in MEF cells, which were defective of \( \text{G}\alpha q \)-11. The amount of protein expressed was verified by Western-blotting (Fig. 4C). As a control, we studied the effects of phenylephrine, which is an agonist at \( \text{\alpha}1 \)-adrenoceptors. As expected, phenylephrine did not increase inositol phosphate accumulation in \( \text{G}\alpha q \)-11-deficient MEF. In contrast, after transfection of \( \text{G}\gamma q \) phenylephrine stimulated inositol phosphate accumulation in cells expressing wild-type \( \text{G}\gamma q \) about 3-fold. Stimulation of inositol phosphate accumulation was lost when the \( \text{G}\gamma q \) mutants G208A or I25A/E26A were transfected. Similarly, PMT potently stimulated inositol phosphate accumulation following expression of wild-type \( \text{G}\gamma q \) in \( \text{G}\alpha q/G\beta\gamma \)-deficient MEFs. As observed for GPCR-mediated effects of phenylephrine, the \( \text{G}\gamma q \) mutants G208A or I25A/E26A did not allow stimulation of inositol phosphate accumulation by PMT-treatment, indicating the essential role of \( \text{G}\gamma \) (Fig. 4B).

![Fig. 4. Mutants of \( \text{G}\gamma q \) affecting \( \text{G}\gamma \) binding, block PMT-induced activation of \( \text{G}\gamma q \)-PLC\(\beta\)) pathway. \( \text{G}\gamma q \), \( \text{G}\gamma q^{208A} \) or \( \text{G}\gamma q^{I25A/E26A} \) encoding retrovirus was produced and \( \text{G}\gamma q \)-\( \text{G}\gamma q^{I25A/E26A} \)-deficient MEF were transduced with resulting retrovirus as described under “Experimental Procedures”. Additional transfection of \( \text{\alpha}1 \)-adrenoceptor expression plasmid was done with the Nucleofection system (Amaxes Biosystems). Transduced MEF were stimulated with 10 \( \mu\)M phenylephrine (PE) for 30 min (panel A) or with 10 nM PMT for 6 h (panel B). The total amount of inositol phosphates was measured as described under “Experimental Procedures”. A, indicates significant difference (\( p<0.005 \)) calculated with ANOVA. Data are given as fold induction over buffer control (mean ± SEM; \( n=3 \)). B. Expression of \( \text{G}\gamma q \), \( \text{G}\gamma q^{208A} \) or \( \text{G}\gamma q^{I25A/E26A} \) in retroviral transduced \( \text{G}\gamma q \)-\( \text{G}\gamma q^{I25A/E26A} \)-deficient MEF was examined by Western-blotting. Shown is an immunoblot of RIPA-extracts of \( \text{G}\gamma q \)-\( \text{G}\gamma q^{I25A/E26A} \)-deficient cells. The immunoblot was performed as described under “Experimental Procedures”.

For this purpose \( \text{Rho-} \) and \( \text{SRF-} \)-dependent luciferase activation was measured in the presence and absence of GRK2-CT. As a control, effects of the muscarinic (M\textsubscript{3}) receptor agonist carbachol were compared with PMT. As shown in Fig. 5A, carbachol- and PMT-induced activation of luciferase activity was strongly reduced by co-expression of GRK2-CT. The GRK-2 mutant (R857Q) of the C-terminal fragment (Fig. S2C), which does not sequester \( \text{G}\gamma \) subunits [37], did not inhibit PMT-induced luciferase activation. Fig. 5B shows the concentration dependency of the inhibiting effect of the C-terminus of GRK2. Increasing expression of GRK2-CT inhibited Rho-dependent luciferase activity stimulated by PMT. In addition, we examined the effect of phosducin, which is another scavenger of \( \text{G}\gamma \) proteins [38–40]. Over-expression of phosducin inhibited muscarinic receptor-mediated SRF-dependent luciferase activity (Fig. 5C and D) and, similarly, increase in luciferase activity caused by PMT treatment of cells was inhibited by phosducin.
This inhibiting effect was observed over a wide concentration range of PMT (Fig. 5D).

4. Discussion

PMT is a potent GPCR-independent activator of Goq and Goi proteins [11]. Here we addressed the question, whether toxin-induced activation of G proteins also results in activation of Gq/12/13. To this end, the activation of P3K, which is an established effector of Gq/12/13 [14,25,26], was analyzed after treatment of cells with PMT. As a read out for activation of P3K, we investigated the translocation of GFP-Grp1php from the cytosol to membranes. Due to its PH domain, the GFP-Grp1php protein binds to PIP3, which is a product of PI3K and is located in the membrane. We show that after over-expression of p110/105 (PI3K), PMT caused a strong and specific translocation of GFP-Grp1php from the cytosol to membranes, indicating the activation of P3K. These findings were corroborated by sequestration experiments with GRK2-CT. The C-terminal part of GRK-2 is known to bind Gi thereby preventing signaling via Gi [28,29]. GRK2-CT effectively blocked PMT-induced translocation of GFP-Grp1ph of PMT, which resulted in inhibition of PMT-induced activation of G protein effects. Therefore, PMT-induced inositol phosphate accumulation and Rho-dependent SRF-activation were studied in the presence of the Gi scavengers GRK2-CT, which resulted in inhibition of PMT-induced activation of G protein effects. Inhibition of PMT-induced effects on G proteins by sequestration of Gi was confirmed by using phosducin, which is another protein that sequesters Gi [38–40].

Two well characterized mutants of Goq (I25A/E26A and G208A) were employed to study the role of Gq/12/13 in more detail. The double mutant Goq(I25A/E26A) is not able to bind Gi [33,41], whereas Goq(G208A) interacts with high affinity with Gi subunits [34–36]. With both...

Fig. 5. Sequestration of Gi subunits inhibits PMT-induced activation of SRE-dependent luciferase expression. A. Luciferase production was measured in HEK293-M3 cells transfected with pSRE,L, pRL.TK and control vectors alone (con), GRK2-CT or GRK2-CT[N587Q] (each 50 ng of DNA per well). Cells were stimulated for 24 h with 1 mM carbachol (Cch, black bars, left part) and 1 nM PMT (black bars, right part) or left unstimulated (white bars). Given is the fold stimulation of luciferase as compared to controls. Data are given as mean ± SEM (n=4)

B. Luciferase production was measured in HEK293-M3 cells transfected with pSRE,L, pRL.TK and increasing amounts of GRK2-CT (μg/12 well). Cells were stimulated for 24 h with 1 mM carbachol (Cch, black bars, left part). 1 nM PMT (black bars, right part) or left unstimulated (white bars). Indicates significant difference between stimulated values (p<0.005) calculated with ANOVA. C. Luciferase production was measured in HEK293-M3 cells transfected with pSRE,L, pRL.TK and control vectors alone (con) or phosducin (Pd, each 50 ng of DNA per well). Cells were stimulated for 24 h with 1 mM carbachol (Cch, black bars, left part), 1 nM PMT (black bars, right part) or left unstimulated (white bars). Indicates significant difference between stimulated values (p<0.005) calculated with ANOVA. D. Luciferase production was measured in HEK293-M3 cells transfected with pSRE,L, pRL.TK and control vectors alone (con, rhombus) or phosducin (Pd, triangle) (each 50 ng of DNA per well). Cells were stimulated for 24 h with increasing concentration of PMT as indicated. Given is the fold stimulation of luciferase as compared to controls. Data are given as mean ± SEM (n=4).
mutants, the PMT-induced activation of \( \alpha_q \) was blocked. These findings suggest that the cycling of \( \gamma \) e.g. the association with and/or dissociation from \( \alpha_q \), is essential for the action of the toxin on \( \alpha \) proteins. This is surprising, because it has been shown that at least the C-terminus of \( \alpha_q \), which is essential for interaction with GPCRs, is not needed for PMT-induced activation of \( \alpha_q \) proteins [11]. Recently we showed that PMT causes inhibition of the GTPase activity and uncouples \( \alpha_q \) from activating receptors [12]. Because \( \gamma \) subunits are not directly involved in the GTPase activity of G proteins, we speculate that it is necessary for loading of the \( \alpha \) protein with GTP. However, our findings cannot exclude that the mutations (e.g., I25A/E26A and G208A) of \( \alpha_q \) affect the interaction of PMT or the interaction of a cellular regulator (e.g. RGS) with the \( \alpha \) protein.

5. Conclusion

Taken together, here we show that PMT-induced activation of heterotrimeric \( \alpha \) proteins leads not only to signaling of the \( \alpha \) subunit but also to release of \( \gamma \). By a pharmacological approach we could show that \( \alpha_q \) is not the only source of \( \gamma \). Also PMT-activated \( \alpha_q \) contributes to \( \gamma \) release. Additionally, we show that \( \gamma \) plays an important role in PMT-induced activation and signaling of the \( \alpha \) proteins.

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Appendix A. Supplementary data


References