Pleiotropic Effects of Pasteurella multocida Toxin Are Mediated by G$_q$-dependent and -independent Mechanisms

IN Volvement of G$_q$ But Not G$_{11}$

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Alexandra Zywietz, Antje Gohla, Milena Schmelz, Günter Schultz, and Stefan Offermanns

From the Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany and the Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67-73, 14195 Berlin, Germany

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§ Present address: Scripps Research Inst., 10550 N. Torrey Pines Rd., La Jolla, CA 92037.
¶ To whom correspondence should be addressed: Pharmakologisches Institut, Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany. Tel.: 49-6221-548246; Fax: 49-6221-548549; E-mail: Stefan.Offermanns@urz.uni-heidelberg.de.

** The abbreviations used are: PMT, P. multocida toxin; DME, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; CNF, cytotoxic necrotizing factor.

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Pasteurella multocida toxin (PMT) is a highly potent mitogen for a variety of cell types. PMT has been shown to induce various cellular signaling processes, and it has been suggested to function through the heterotrimeric G-proteins G$_q$/G$_{11}$. To analyze the role of G$_q$/G$_{11}$ in the action of PMT, we have studied the effect of the toxin in G$_q$/G$_{11}$ double-deficient fibroblasts as well as in fibroblasts lacking only G$_q$ or G$_{11}$. Interestingly, formation of inositol phosphates in response to PMT was exclusively dependent on G$_q$, but not on the closely related G$_{11}$. Although G$_q$/G$_{11}$ double-deficient and G$_q$-deficient cells did not respond with any production of inositol phosphates to PMT, PMT was still able to induce various other cellular effects in these cells, including the activation of Rho, the Rho-dependent formation of actin stress fibers and focal adhesions, as well as the stimulation of c-Jun N-terminal kinase and extracellular signal-regulated kinase. These data show that PMT leads to a variety of cellular effects that are mediated only by part of the heterotrimeric G-protein G$_q$.

Pasteurella multocida is a small Gram-negative cocobacillus present in the nasooropharynx and gastrointestinal tract of many avian and mammalian species. Infections of P. multocida are associated with atrophic rhinitis in pigs (1) and dermatonecrosis, respiratory disease in cattle and rabbits (2), and dermatonecrosis and bacteremia in humans (3, 4). P. multocida produces a 146-kDa protein that is its major pathogenic factor (5–7). P. multocida toxin (PMT) has little homology to C. botulinum exoenzyme C5 (8–10). Exposure of cells to PMT results in tyrosine phosphorylation of various proteins including focal adhesion kinase and paxillin as well as in actin stress fiber formation and focal contact assembly (9, 12, 13). Several lines of evidence suggest that some of these effects are mediated by the small GTP-binding protein Rho, which plays a major role in actin cytoskeleton dynamics (14). Disruption of Rho function by the C3 exoenzyme of Clostridium botulinum, which ADP-ribosylates and thereby inhibits Rho abolishes focal contact formation in response to PMT, and incubation of endothelial cells with C3 exoenzyme blocks PMT-induced actin stress fiber formation (12, 13).

PMT has also been shown to induce a robust increase in inositol phosphate levels, mobilization of intracellularly stored calcium, production of diacylglycerol, and activation of protein kinase C, suggesting that it leads to an activation of phospholipase C (10, 15–18). PMT potentiates the production of inositol phosphates induced by various agonists that function through receptors coupling to G-proteins of the G$_q$/G$_{11}$ family, and PMT-induced formation of inositol phosphates can be inhibited by guanosine 5′-O-(β-thiodiphosphate) (18). It has therefore been proposed that PMT-induced G-proteins of the G$_{11}$ family may be involved in the action of PMT. The G$_{11}$ family contains four members, of which two, G$_q$ and G$_{11}$, are expressed in almost all tissues of the mammalian organism and couple heptahelical receptors in a stimulatory fashion to β-isofoms of phospholipase C (18). Further evidence for a possible role of G$_q$/G$_{11}$ in cellular effects of PMT came from studies in Xenopus oocytes. A PMT-induced Ca$^{2+}$-dependent chloride current could be suppressed by injection of a G$_{11}$ antisense RNA and an antiserum recognizing both G$_{11}$ and G$_{11}$ (20). In addition, PMT-induced phosphorylation of ERK-1 was reduced by expression of a C-terminal peptide of G$_{11}$ that is believed to interfere with receptor-G$_{11}$ interaction (21).

To determine the exact role of G$_q$/G$_{11}$ in various cellular responses of PMT, we have studied the effect of PMT in G$_q$/G$_{11}$ double-deficient fibroblasts as well as in fibroblasts lacking only G$_q$ or G$_{11}$. Surprisingly, we found that the formation of inositol phosphates in response to PMT is dependent on G$_q$, but not on the closely related G$_{11}$. In addition, exposure of cells to PMT induced Rho activation, Rho-dependent stress fiber formation, and activation of MAP kinases in a manner independent of G$_q$/G$_{11}$. These data show that PMT leads to pleiotropic effects in a G$_q$-dependent and -independent manner.

EXPERIMENTAL PROCEDURES

Materials—Y-27632 was provided by Yoshitomi Pharmaceutical Industries. The mutated Rho-binding domain of Rho kinase, RB/PH(PT), was a gift from K. Kaibuchi (Ikoma, Japan). C. botulinum C3-exoen-
zyme was a donation from I. Just and K. Aktories (Freiburg, Germany) or was purchased from Upstate Biotechnology. PMT was purchased from Sigma.

Cell Culture—Wild-type fibroblasts and fibroblasts lacking both G-protein α-subunits were derived from embryonic day 10.5 mouse embryos originating from intercrosses of Gαq(−/−) and Gα11(−/−) mice. The generation of Gαq and Gα11 mutant mice has been described previously (22, 23). Fibroblasts lacking G-protein α-subunits were prepared and cultured as described previously (24).

Microinjection—For microinjection studies, cells were seeded at a density of ~105 cells/mm2 on glass coverslips imprinted with squares to facilitate the localization of injected cells and grown overnight. To obtain quiescent and serum-starved fibroblasts, cultures were rinsed in serum-free DMEM and incubated in DMEM supplemented with 25% Ham’s F-12 medium, 0.2% NaHCO3, 10 mM Hepes, and 0.1% fetal bovine serum (modified DMEM) for 24 h, followed by a 48-h incubation in modified DMEM devoid of fetal bovine serum. Plasmids were injected into the nucleus together with Texas Red dextran (5 mg/ml; Molecular Probes) to visualize injected cells. C. botulinum C3 exoenzyme was coinjected with the CDNAs at a concentration of 100 μg/ml. About 150 cells/field were injected in each case, using a manual injection system (Eppendorf, Hamburg, Germany).

Visualization of Actin Cytoskeleton—Microinjected cells were stimulated with 100 ng/ml PMT overnight, fixed in 4% paraformaldehyde for 20 min, and permeabilized in 0.2% Triton X-100 for 5 min. To visualize the actin cytoskeleton, cells were stained for polymerized actin by incubation with 0.5 μg/ml fluorescent isothiocyanate-phallolidin (Sigma) for 40 min. The coverslips were mounted on glass slides and examined using an inverted microscope (Zeiss Axiovert 100). Quantification of actin stress fibers was performed as described (24).

Determination of Inositol Phosphate Levels—Cells were labeled for 20–24 h with 120 pmol of myo-[3H]inositol (758.5 GBq/mole; PerkinElmer Life Sciences) well in the absence or presence of PMT. For determination of receptor-mediated inositol phosphate production, cells were washed with inositol-free medium and then incubated for 10 min at 37°C with 0.25 ml of inositol-free medium containing 10 mM LiCl. Thereafter, medium was aspirated, the indicated agents were added in medium containing 10 mM LiCl, and cells were incubated for 20 min. Inositol phosphate production was stopped by addition of 0.2 ml of 10 mM ice-cold formic acid. After keeping the samples on ice for 20 min, and permeabilized in 0.2% Triton X-100 for 5 min. To visualize the actin cytoskeleton, cells were stained for polymerized actin by incubation with 0.5 μg/ml fluorescent isothiocyanate-phallolidin (Sigma) for 40 min. The coverslips were mounted on glass slides and examined using an inverted microscope (Zeiss Axiovert 100). Quantification of actin stress fibers was performed as described (24).

Determination of Activated Cellular RhoA—The amount of activated cellular RhoA was determined by precipitation with a fusion protein consisting of GST and the Rho-binding domain of Rhotekin (amino acids 7–89; GST-Rho-binding domain) as described (26). Cells were washed with ice-cold Hank’s buffer and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 10 μM/ml each of leupeptin and aprotime, and 1 mM PMSF). Clarified cell lysates were incubated with GST-Rho-binding domain (20 μg of beads) at 4°C for 45 min. The beads were washed four times as described (26), and the precipitated RhoA was detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

Determination of ERK Phosphorylation and c-Jun Kinase Activity—For determination of ERK phosphorylation, serum-starved (48 h 0.5% fetal calf serum, 24 h 0.1% fetal calf serum) cells grown in 12-well dishes were washed once with phosphate-buffered saline and lysed in Laemmli sample buffer. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and phosphorylation of ERK was determined by immunoblotting with an anti-phospho-ERK antiserum (New England Biolabs). Blots were reprobed with an anti-ERK antiserum (New England Biolabs). c-Jun kinase activity was determined in a solid phase assay using GST-c-Jun as a substrate (27, 28). GST-c-Jun phosphorylated in the presence of [γ-32P]ATP was subjected to SDS-polyacrylamide gel electrophoresis, and phosphorylation of c-Jun was determined by autoradiography of dried gels (29). JNK1 and JNK2 were detected with an anti-JNK-antiserum (Santa Cruz Biotechnologies).

RESULTS
For studies on the possible role of Gαq/Gα11 in the cellular effects of PMT, we employed fibroblast cell lines derived from mouse embryos deficient in either Gαq or Gα11 and lacking both G-protein α-subunits. The absence or presence of Gαq and Gα11 was verified by immunoblotting (Fig. 1). Treatment of wild-type mouse fibroblasts with 100 ng/ml PMT for increasing time periods resulted in a marked and time-dependent accumulation of inositol phosphates that could be observed 8 h after addition of the toxin and reached a maximum after about 20 h (Fig. 2A). In contrast, incubation of Gαq/Gα11 double-deficient fibroblasts for various time periods did not result in any increase in the formation of inositol phosphates (Fig. 2A). Inositol phosphate production in wild-type cells could be induced with 10 ng/ml PMT and increased dose-dependently up to a concentration of 1000 ng/ml of the toxin (Fig. 2B). However, even at PMT concentrations that were maximally effective in wild-type fibroblasts, no effect on inositol phosphate levels could be observed in Gαq/Gα11 double-deficient fibroblasts (Fig. 2B). This indicates that G-proteins of the Gαq/Gα11-family are indeed required for PMT-induced inositol phosphate formation.

The α-subunits of Gαq and Gα11 are highly homologous, and so far no functional differences between Gαq and Gα11 either with regard to the activation through receptors or their regulation of effectors have been reported. To test whether both Gαq and Gα11 are involved in PMT-induced formation of inositol phosphates, we tested the effect of PMT on inositol phosphate production in cells that lack only Gαq or Gα11 (Fig. 3). The expression of either Gαq or Gα11 was sufficient to mediate receptordependent phospholipase C activation because inositol phosphate production could be induced by thrombin and Bradykinin in Gαq-deficient cells like Gαq/Gα11 as well as in Gαq and Gα11 double-deficient cells, but not in Gαq/Gα11 double-deficient fibroblasts (Fig. 3). However, although Gα11-deficient fibroblasts still responded with an increased inositol phosphate production to PMT, Gαq-deficient cells behaved like Gαq/Gα11 double-deficient fibroblasts and were completely unresponsive, indicating that the effect of PMT was mediated solely by Gαq and not by Gα11.

PMT has also been shown to induce a Rho-dependent actin stress fiber formation and focal adhesion formation in fibroblasts and endothelial cells (9, 12, 13). There are conflicting data as to the ability of Gαq/Gα11 to induce the formation of actin stress fibers (30, 31). However, in some systems Gαq/Gα11 have been shown to be able to regulate Rho-dependent processes (32–34). To study the involvement of Gαq/Gα11 in PMT-induced actin stress fiber formation and focal adhesion assembly, we tested the effect of the toxin on the actin cytoskeleton in wild-type and Gαq/Gα11 double-deficient fibroblasts (Fig. 4). Actin filaments were visualized by fluorescein isothiocyanate-labeled phallolidin, and focal adhesions were stained with an anti-vinculin antibody. In serum-starved fibroblasts lacking Gαq and Gα11, PMT induced a pronounced formation of actin stress

![FIG. 1. Expression of Gαq and Gα11 in fibroblasts derived from Gα-deficient mouse embryos.](http://www.jbc.org/)

![FIG. 2. Inositol phosphate levels.](http://www.jbc.org/)
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PMT (or for 20 min with a mixture of 5 units/ml thrombin (or intranuclearly with an expression plasmid carrying a dominant negative Rho kinase (K) shortly before exposure to PMT was started. To detect injected cells, cells were coinjected with Texas Red-labeled dextran. Injected cells are marked with arrows.

FIG. 3. Effect of PMT and thrombin/bradykinin on inositol phosphate production in embryonic fibroblasts lacking Goq and/or Goq. Wild-type cells, Goq, Goq11, or Goq/Goq11 double-deficient cells were incubated for 20 h in the absence or presence of 100 ng/ml PMT (upper panel) or for 20 min with a mixture of 5 μM bradykinin and 5 units/ml thrombin (lower panel). PMT-dependent inositol phosphate production was determined as described under "Experimental Procedures." Shown are the mean values of triplicates ± S.D.

fibers and a formation of focal adhesions indistinguishable from its effect in wild-type cells (Fig. 4). This indicates that the PMT-induced reorganization of the actin cytoskeleton and focal adhesion assembly occurred independently of the Gq/G11-mediated signaling pathway. Preincubation of cells with pertussis toxin did not affect PMT-induced actin stress fiber formation, indicating that G-proteins of the Gi family were not involved. Induction of actin stress fiber formation through various receptors in fibroblasts has been shown to involve a Rho/Rho kinase-mediated signaling pathway (35–37). The actin stress fiber formation by PMT in Goq/Goq11 double-deficient fibroblasts could be blocked by cytosolic injection of C3 exoenzyme of C. botulinum, which ADP-ribosylates Rho at residue Asn41 in its effector domain resulting in the inactivation of Rho (38) (Figs. 4 and 5). Intranuclear injection of an expression plasmid carrying a dominant negative mutant of Rho kinase comprising a mutated Rho-binding and adjacent pleckstrin homology domain of Rho kinase, RB/PH(TT) (39), and preincubation of cells with the Rho kinase inhibitor Y-27632 (40) strongly inhibited PMT-induced actin stress fiber formation (Figs. 4 and 5). These data suggest that PMT engages a Rho/Rho kinase-mediated signaling pathway to induce actin stress fiber formation.

To test whether PMT indeed induces the activation of Rho, we directly determined Rho activation by precipitation of endogenous GTP-bound Rho using a fusion protein consisting of glutathione S-transferase and the Rho-binding domain of rhodopsin (26). As shown in Fig. 6 PMT induced a pronounced activation of Rho that was indistinguishable in wild-type and Goq/Goq11 double-deficient cells, indicating that PMT-induced Rho activation is independent of the Gq/G11-dependent signaling pathway. Similar to the Gq-mediated PMT-induced inositol phosphate production, the Gq/G11-independent activation of Rho could only be observed after a lag period of several hours following exposure of cells to PMT (data not shown).

Various Gq family members have been demonstrated to mediate the activation of JNK and ERK (41–46), and constitutively active mutants of Gq-family members have been shown to stimulate JNK activity in some cellular systems (47, 48). In addition, PMT has been shown to induce stimulation of ERK, an effect that could be inhibited by a dominant negative Goq mutant (21). To delineate the role of Goq/Goq11 in PMT-induced MAP kinase activation, we compared its effect on JNK and

FIG. 2. Effect of PMT on inositol phosphate production in wild-type and Goq/Goq11-deficient embryonic fibroblasts. A, wild-type cells (closed circles) and Goq/Goq11-deficient cells (open circles) were incubated for the indicated time periods (abscissa) in the presence of 100 ng/ml PMT. B, wild-type cells (closed circles) and Goq/Goq11-deficient cells (open circles) were incubated for 16 h with the indicated concentrations of PMT. PMT-dependent inositol phosphate production was determined as described under "Experimental Procedures." Shown are the mean values of triplicates ± S.D.
bleasts were injected cytoplastically with 100 ng/ml C3-exoenzyme (C3) or intranuclearly with an expression plasmid carrying a dominant negative Rho kinase (dnROCK) or were treated with pertussis toxin (100 ng/ml for 16 h; PTX) or with Y-27632 (10 μM for 12 h before fixation). Injected cells were detected by coinjection of a fluorescent dye. After exposure to 100 ng/ml of PMT for 16 h, cells were fixed, and actin stress fibers were visualized with fluorescein isothiocyanate-phalloidin. Shown is the percentage of stress fiber positive cells. In each case, at least 120 cells were analyzed.

ERK activity in wild-type and \( \alpha_q/\alpha_{11} \)-double-deficient fibroblasts (Fig. 7). PMT induced activation of JNK as well as of ERK in both wild-type and \( \alpha_q/\alpha_{11} \)-double-deficient fibroblasts, demonstrating that PMT leads to JNK and ERK activation in a manner independent of \( \alpha_q \).

**DISCUSSION**

PMT has been shown to induce a variety of cellular effects. The precise molecular mechanism by which PMT acts is, however, still poorly defined. The toxin has only moderate homology to other proteins, and so far no enzymatic activity has been detected. Similar to many other toxins, PMT requires internalization and intracellular processing to exhibit cellular effects. This results in a lag period of a few hours between exposure of PMT to intact cells and the occurrence of cellular changes (16). It has been suggested that G-proteins of the \( \alpha_{11} \) family are involved in the cellular action of PMT. The two main members of this family, \( \alpha_q \) and \( \alpha_{11} \), are structurally and functionally highly homologous and couple receptors in a stimulatory fashion to \( \beta \)-isoforms of phospholipase C (19, 49). An antiserum recognizing the \( \alpha \)-subunits of both \( \alpha_q \) and \( \alpha_{11} \) blocks a PMT-induced Ca\(^{2+} \)-dependent Cl\(^- \) current in Xenopus oocytes, which involves PLC-\( \beta \) (20). Stimulation of this current by PMT could also be inhibited by the injection of \( \alpha_q \) antiserum RNA, whereas sense RNA potentiated the effect of PMT (20). These data were obtained after injection of PMT into oocytes, which results in a rapid response within seconds after injection. This, however, is a situation completely different from the action of the toxin on intact cells that requires internalization and processing of PMT. In a recent study using HEK-293 cells, it was shown that PMT-induced activation of Erk-1 can be reduced by about 70–80% upon expression of a C-terminal fragment of \( \alpha_q \) that is supposed to act in a dominant negative fashion (21). Although these studies support an involvement of \( \alpha_q/\alpha_{11} \) in some of the cellular effects of PMT, the evidence provided remains indirect.

To study the role of G-proteins of the \( \alpha_q/\alpha_{11} \) family in the cellular action of PMT, we used fibroblast cell lines derived from mouse embryos that are deficient in \( \alpha_q/\alpha_{11} \). In wild-type embryonic fibroblasts PMT induced a robust time- and dose-dependent increase in the production of inositol phosphates that could not be observed in fibroblasts lacking both \( \alpha_q \) and \( \alpha_{11} \) (Figs. 2 and 3). Embryonic fibroblasts lacking only \( \alpha_q \) did not respond to PMT with inositol phosphate production, whereas PMT lead to a strong response in \( \alpha_{11} \)-deficient cells, indicating that PMT-induced inositol phosphate production is mediated by \( \alpha_q \) and not by \( \alpha_{11} \). This is surprising because evidence collected from biochemical, pharmacological, and somatic cell genetic studies suggested that \( \alpha_q \) and \( \alpha_{11} \) have very similar, if not identical, characteristics. \( \alpha_q \) and \( \alpha_{11} \) couple to the same set of seven transmembrane receptors with the same effector specificity for phospholipase C-\( \beta \) isoforms (50–54).

Although our results in \( \alpha_q/\alpha_{11} \)-deficient cells clearly show that the \( \alpha_q/\beta \) pathway plays an important role in the action of PMT, it has been suggested that PMT can also act independently of PLC-mediated Ca\(^{2+} \) mobilization and protein kinase C activation (12). We therefore tested whether PMT can
still induce cellular effects in the absence of Gq-dependent signaling. Exposure of Gaq/G11-deficient embryonic fibroblasts to PMT resulted in actin stress fiber formation and focal adhesion assembly (Figs. 4 and 5). Actin stress fiber formation was inhibited by C3 exoenzyme of C. botulinum as well as by a dominant negative form of Rho kinase and the Rho kinase inhibitor Y-27632. This indicates that a Rho/Rho kinase mediated but Gaq/PLC-β-independent pathway is involved in this cellular response to PMT. A Rho/Rho kinase-mediated pathway resulting in the inhibition of myosin phosphatase and subsequent increase in myosin light chain phosphorylation has recently been proposed to underlie PMT-induced reorganization of the actin cytoskeleton in endothelial cells (13). Actin rearrangement induced by PMT in endothelial cells could be completely blocked by an inhibitor of the Ca2+/calmodulin-regulated myosin light chain kinase, suggesting that dual regulation of myosin light chain phosphorylation through Ca2+-dependent myosin light chain kinase activation and Rho/Rho kinase-mediated myosin phosphatase inhibition is involved in the effect of PMT on the actin cytoskeleton. Our data, however, suggest that the inhibition of myosin phosphatase through Rho/Rho kinase is sufficient to induce a rearrangement of the actin cytoskeleton in embryonic fibroblasts because it can be observed in the absence of a Gaq-mediated inositol phosphate production and subsequent Ca2+ mobilization.

The involvement of Rho in PMT-induced cellular effects could be directly demonstrated by precipitation of active Rho from cell lysates of PMT-exposed wild-type and Gaq/G11-deficient embryonic fibroblasts. Thus, activation of Rho by PMT occurs independently of Gaq/G11. The N-terminal half of cytotoxic necrotizing factor (CNF) 1 and 2 from Escherichia coli show moderate homology with N-terminal regions of PMT, and CNF1 has been shown to inhibit the GTPase activity of RhoA by deamidation of glutamine residue 63 resulting in constitutive activation of Rho (55, 56). However, the catalytic activity of CNF1 appears to reside in the C-terminal part of the toxin (57, 58), and it is unlikely that PMT functions analogously to CNF1 (12, 13, 20). PMT may act upstream of Rho by regulating the activity of a guanine nucleotide exchange factor or a GTPase-activating protein specific for Rho. G-proteins of the G12 family that have been shown to be able to mediate Rho activation (30, 59, 60) are not involved because PMT-induced Rho activation could also be observed in fibroblasts lacking Gaq/G12/G13 (data not shown).

Various MAP kinases including JNK and ERK have been shown to be regulated through Gq/G11-coupled receptors, and PMT has been reported to activate ERK via a pathway involving the epidermal growth factor receptor (21). Incubation of wild-type fibroblasts with PMT resulted in a clear JNK and ERK activation. This effect of PMT obviously did not involve Gq/G11 because Gaq/G11-deficient cells also responded with activation of JNK and ERK after exposure to PMT to a comparable extent as did wild-type cells (Fig. 7). It has previously been shown that PMT-induced ERK activation is mediated by Gaq/G11 (21). Our data demonstrate that Gaq/G11 are not required for JNK and ERK activation by PMT in embryonic fibroblasts. However, we cannot exclude the possibility that Gaq contributes to PMT-induced MAP kinase activation in wild-type cells.

In summary, we show that PMT induces a remarkable array of cellular effects including the activation of phospholipase C, which is entirely dependent on Gaq but not on the closely related G-protein G11. However, the pleiotropic actions of PMT are only in part mediated by Gaq. Activation of Rho and MAP kinases can be induced by PMT in a Gaq/G11-independent manner, suggest-
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