Modification of Heterotrimeric G-Proteins in Swiss 3T3 Cells Stimulated with Pasteurella multocida Toxin

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Abstract

Many bacterial toxins covalently modify components of eukaryotic signalling pathways in a highly specific manner, and can be used as powerful tools to decipher the function of their molecular target(s). The Pasteurella multocida toxin (PMT) mediates its cellular effects through the activation of members of three of the four heterotrimeric G-protein families, Gq, G12 and Gi. PMT has been shown by others to lead to the deamidation of recombinant Gq11 at Gln-205 to inhibit its intrinsic GTPase activity. We have investigated modification of native Gα subunits mediated by PMT in Swiss 3T3 cells using 2-D gel electrophoresis and antibody detection. An acidic change in the isoelectric point was observed for the Gαq and Gαi subunits of the Gq and Gi families following PMT treatment of Swiss 3T3 cells, which is consistent with the deamidation of these Gα subunits. Surprisingly, PMT also induced a similar modification of Gα13, a member of the Gq family of G-proteins that is not activated by PMT. Furthermore, an alkaline change in the isoelectric point of Gα13 was observed following PMT treatment of cells, suggesting differential modification of this Gα subunit by PMT. Gα11 was not affected by PMT treatment. Prolonged treatment with PMT led to a reduction in membrane-associated Gαq, but not Gαq. We also show that PMT inhibits the GTPase activity of Gαq.

Introduction

Heterotrimeric G-proteins are a family of key signal transduction proteins that intercede between the many G-protein coupled receptors (GPCR) that the cell uses to interrogate its local environment and downstream signalling pathways that ultimately regulate fundamental cellular choices [1]. G-proteins are divided into 4 classes (Gq, G12, Gi and Gs) according to their constituent subunits [2]. Activation of a GPCR causes a conformational change in its cognate Gα subunit, which is a guanine nucleotide binding protein that can exist in an inactive GDP-bound or an active GTP-bound form [3]. Activation of a GPCR causes a conformational change in its cognate Gα subunit that triggers GDP to be exchanged for GTP. The activated state persists until GTP is hydrolysed to GDP by the intrinsic GTPase activity of the Gα subunit. G-proteins are also subject to reversible tyrosine phosphorylation and lipid modifications during their activation cycle, but the regulatory role of these events is not fully understood [3]. Each G-protein class activates a characteristic set of downstream targets. The Gq and Gi families activate or inhibit adenylate cyclase, respectively [4]. The Gq family activates phospholipase C (PLC) [5], while the Gi family is particularly linked to activation of the Rho GTPase [6].

Intracellularly-acting bacterial protein toxins enzymatically modify a limited and precise set of cellular proteins to modulate their function. The Pasteurella multocida toxin (PMT) activates multiple signalling pathways in cultured cells leading characteristically to a strong mitogenic response [7]. PMT has been shown to activate members of the Gq, G12, and Gi families [8–13]. PMT catalyses the deamidation of recombinant Gα at Gln-205 to inhibit its intrinsic GTPase activity [14]. We describe here the effects of PMT on all four classes of heterotrimeric G-proteins in Swiss 3T3 cells using two-dimensional (2-D) gel electrophoresis and other techniques.

Materials and Methods

Reagents

Cell culture reagents were obtained from Invitrogen. [γ-32P] GTP was obtained from PerkinElmer LAS. Anti-Gαq(11-14) (sc-392), anti-Gαq(13) (sc-394), anti-Gα11 (sc-387), anti-Gα15 (sc-410) and anti-Gα2 (internal: sc-7276) antibodies were from Santa Cruz Biotechnology. Anti-Gαq(371752), anti-Gαq(13) (371720), anti-Gαq(1-2) (371723) and anti-Gα13(1-3) (371729: which is known to cross react with Gαq and Gα2) antibodies were purchased from Calbiochem-Novabiochem. Phospho-FAK (Tyr397) was from New England Biolabs Ltd. All reagents used for 2-D gel electrophoresis were from GE HealthCare, unless otherwise stated. Recombinant PMT was purified essentially as described [15]. A recombinant His-tagged Gαq subunit (371765) was purchased from Calbiochem-Novabiochem. Recombinant His6-tagged human Gα11 was expressed and purified from E. coli containing pProEX-HTb, which was provided as a kind gift by Professor David Siderovsky (Department of Pharmacology, University of North Carolina, ...
USA) [16]. All other chemical reagents were of analytical grade and were obtained from Sigma-Aldrich, unless otherwise stated.

Cell culture

Swiss 3T3 cells, originally developed by Todaro and Green [17], and kindly provided by Theresa Higgins (Cancer Research UK, London, UK) were cultured as described [9]. Cells were grown to confluence and used when quiescent, before the addition of PMT or bombesin (Calbiochem-Novabiochem). The tyrosine kinase inhibitors Su6656 and Su6638 (Calbiochem-Novabiochem) were prepared in DMSO, diluted in DMEM containing 0.1% DMSO and added to cell cultures to give a final concentration of 100 μM 1 h prior to treatment with PMT.

Preparation of Swiss 3T3 membranes and cytoplasmic fractions

Swiss 3T3 cells were grown in 145 mm dishes, rinsed twice with ice cold PBS and scraped into 2 ml of PBS containing protease inhibitors (Complete, Roche Diagnostics). Cells from 10 dishes were pooled, collected by centrifugation (200 g, 10 min, 4°C), and washed cell pastes were frozen at −70°C until required. The frozen cell pastes (~5 mg) were thawed on ice and suspended in 5 ml of membrane buffer (10 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.4, containing protease inhibitors). The cells were ruptured by 25 passes through a 23-gauge needle, and the resulting homogenate was centrifuged at 800 g for 10 min to remove unbroken cells and nuclei. The supernatants were transferred to fresh tubes, and centrifuged at 50,000 g for 10 min. The supernatant containing cytoplasmic proteins was transferred to fresh tubes and centrifuged at 50,000 g for 10 min. The supernatant containing cytoplasmic proteins was transferred to a fresh tube, treated with 4 μl of trypsin mixture (100 μM GDP, 1.5 μg/ml trypsin in solubilisation buffer) for 30 min at 30°C. The trypsin activity was neutralised with 3 μl of soybean trypsin inhibitor (3 mg/ml). Trypsin-resistant fragments were resolved by SDS-PAGE, and detected by immunoblotting using antiserum against Gαq/11. The induction of trypsin protection by GTPαS and GTP alone or in the presence of bombesin or PMT were quantified relative to untrypsinised Gα, using scanning densitometry (Gene-Tools, Syngene). Data were analysed using factorial analysis of variance (ANOVA) by Dr Ron Wilson (King’s College London).

Results

PMT stimulates an acidic modification of Gαq and Gα11 family proteins

Gαq/11 antisemur detected both Gαq and Gα11 subunits at an apparent molecular mass of 42 kDa in membranes prepared from quiescent Swiss 3T3 cells. Separating these subunits on a urea gel showed that Gαq (which aberrantly runs slower in this system than Gα11 [24]) was more abundantly expressed than Gα11 in these cells (Fig. 1A). A similar relative abundance has been shown in rat neurons [25]. Four distinct Gαq/11 molecular isoforms, designated q-II, q-III, q-V and q-VI, were resolved by 2-D gel electrophoresis followed by immunoblotting with anti-Gαq/11 antibody in membranes derived from untreated cells (Fig. 1B). These plus two additional isoforms, q-I and q-IV, were resolved by 2-D PAGE and Western blot analysis of membrane fractions derived from cells treated with PMT (150 pM) for 4 h (Fig. 1C; Table 1).

Calcium microfluorimetry

Intracellular calcium was recorded as given previously [20]. Briefly, Swiss 3T3 cells were plated onto 19 mm glass cover slips and incubated in 5 μM Indo – AM (1 hour, 37°C, in the dark, Calbiochem). Cover slips were placed in a custom built chamber allowing gravity fed superfusion (10–12 ml/min) of a modified Krebs solution. Bombesin was applied by switching a multiway tap to a solution containing it and was removed by switching back to a bombesin free solution. The waste was removed by a peristaltic pump. Recordings were performed at room temperature by subtraction of background light and recording the emitted light from individual cells at 405 and 480 nm. The emission ratio (R) was converted to a calcium concentration after calibration (see reference 20) in which [Ca²⁺] (nM) = 1020(R-0.96)/(12-R) and autofluorescence was less than 4%.

Trypsin protection assay

The trypsin protection assay was adapted from Evanko et al. [21]. Briefly, membrane fractions (100 μg) were incubated with PMT, bombesin, GTPγS or GTP at the required concentrations at 37°C for times indicated. Membrane fractions were centrifuged at 10,000 x g for 10 min at 4°C and the pellet was resuspended in 12.8 μl of solubilisation buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1% Gd₂EG₃ (polyoxyethylene 10-lauryl ether), 0.1 mM phenylmethylsulfonyl fluoride), vortexed, incubated on ice for 20 min and centrifuged at 18,000 x g for 10 min at 4°C. The supernatant was then transferred to a new microfuge tube, treated with 4 μl of trypsin mixture (100 μM GDP, 1.5 μg/ml trypsin in solubilisation buffer) for 30 min at 30°C. The trypsin activity was neutralised with 3 μl of soybean trypsin inhibitor (3 mg/ml). Trypsin-resistant fragments were resolved by SDS-PAGE, and detected by immunoblotting using antisemur against Gαq/11. The induction of trypsin protection by GTPγS and GTP alone or in the presence of bombesin or PMT were quantified relative to untrypsinised Gα, using scanning densitometry (Gene-Tools, Syngene). Data were analysed using factorial analysis of variance (ANOVA) by Dr Ron Wilson (King’s College London). Unactivated Gα subunits (GDP-bound) are highly susceptible to trypsin digestion; however trypsin cleavage is inhibited when G-proteins are activated (GTP-bound) as most cleavage sites are conformationally protected, and a product resulting from a small N-terminal cleavage can be visualised [22].
react with a recombinant Gαq subunit. The Gαq but not the Gα11
antiserum could detect the Gα11 subunit (Fig. 1H). The experimen-
tially determined pI values for the Gαq/11 isoforms (Table 1)
are similar to the predicted pI values of 5.48 and 5.70 for murine
Gαq and Gα11, respectively [26,27].

The expression of the Gαq,-1, Gαq,-2 and Gαq,-3 subclasses, which
have the widest tissue expression pattern of this family [28], was
analysed in Swiss 3T3 cells using specific antisera. The Gαq,-2
(directed against Gαq,-1 and Gαq,-2) and Gαq,-3 antisera (directed
against Gαq,-1, Gαq,-2 and Gαq,-3) each detected an abundant protein
band at 40 kDa in membranes from Swiss 3T3 cells. The
antisem specific for only Gαq,-1 detected a weak band (Fig. 2A),
although this antiserum could be shown to react strongly with a
recombinant Gαq,-1 subunit (Fig. 2B), demonstrating a low
abundance of Gαq,-1 in Swiss 3T3 cells.

Gαq,-1 isoforms were present at low abundance in membranes
prepared from either untreated or PMT-treated Swiss 3T3 cells as
determined by 2-D gel electrophoresis followed by immunoblot-
tting (Fig. 2C, D; Table 2). The Gαq,-1-3 antisem detected two Gαq,
isoforms in untreated and PMT-treated cells, designated i-I and i-
II (Fig. 2E, F; Table 2), with a reproducible change in the relative
abundance of the isoforms after PMT treatment. The Gαq,-1-3
antisem detected 3 Gαq isoforms in untreated cells, two of which
appeared to correspond to i-I and i-II; the third isoform was
designated i-IV (Fig. 2G; Table 2). The Gαq,-1-3 antisem also
detected these and one additional isoform, i-III in PMT-treated
cells (Fig. 2H; Table 2).

The predicted pI values of murine Gαq,-1, Gαq,-2 and Gαq,-3 are
5.69, 5.28 and 5.50, respectively [29]. It seems probable that
isoforms i-I and i-II detected by the Gαq,-2 antisem belong to the
Gαq,-2 subclass, as isoforms of the Gαq,-1 subclass are expected to
have a more basic pI, and Gαq,-1 was not detected in Swiss 3T3
cells. Isoforms i-III and i-IV are therefore likely to belong to the
Gαq,-3 subclass. Orth et al. resolved Gαq,-1 and Gαq,-2 from mouse
embryonic fibroblast cells by 2-D gel electrophoresis at an
unspecified pI value and showed that PMT treatment of these
cells caused an acidic pI shift consistent with deamidated
recombinant Gαq,-2 [14]. Our results suggest that PMT catalyses
the acidic covalent modification of Gαq,-2 and Gαq,-3.

**PMT induces an alkaline modification of Gα13**

The two members of the Gα12 family, Gα12 and Gα13, are
ubiquitously expressed [30]. Gα13 was detected in Swiss 3T3
membranes using antiserum against Gα13 (Fig. 3A). Three Gα13
isoforms, 13-I, 13-III and 13-IV, were identified in membranes
from Swiss 3T3 cells (Fig. 3B). Two additional isoforms, 13-II and
13-V, were detected in membranes derived from PMT-treated
cells (Fig. 3C; Table 3). The additional Gα13 isoforms seem to be
the result of an alkaline pH shift, in contrast to the effect of PMT
on Gαq/11 and Gαq isoforms. Under our experimental conditions,
Gα12 could not be resolved by 2-D gel electrophoresis.

**PMT does not induce any modification of Gαs**

The alpha subunits of the ubiquitously expressed Gα family can
be expressed as four distinct forms as a result of alternative mRNA
splicing [31]. Swiss 3T3 cells were shown to express both large
(55 kDa) and small (52 kDa) forms of Gαs, with Gαs-large being
more abundantly expressed than Gαs-small (Fig. S1A). Six
isoforms of Gαs-large (s-I to s-VI) and two isoforms of Gαs-small
(s-VII and s-VIII) were resolved in membranes derived from Swiss
3T3 cells by 2-D gel electrophoresis, followed by immunoblott-
ing with the Gαs-large antisem (Fig. S1B; Table S1). The Gαs-large
isoforms were detected at a more acidic pI than the Gαs-small
isoforms, which concurs with previous findings [19]. PMT showed

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Figure 1. PMT induces the covalent modification of Gαq and
Gα11. (A) Membrane proteins from Swiss 3T3 cells were separated by
urea gel electrophoresis and Western blotted with anti-Gαq/11 antibody.
The locations of Gαq,-1 and Gαq,-11 are indicated by arrows. Membrane
proteins from Swiss 3T3 cells (B, D, F) left untreated or (C, E, G) treated
with 150 μM PMT for 4 h were separated by 2-D gel electrophoresis and
Western blotted with (B, C) anti-Gαq/11, (D, E) anti-Gαq,-1 or (F, G) anti-Gαq,-11
antibody. (H) Recombinant Gαq subunit (lane 1) and membrane
proteins from Swiss 3T3 cells (lane 2) were separated by SDS PAGE
and Western blotted with anti-Gαq (left panel) or -Gα11 (right panel)
antibody. Samples from at least 3 independent experiments were
resolved with similar results.
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isoforms in untreated cells with pI values corresponding to the
isoforms q-V and q-VI (Fig. 1F; Table 1) and one additional
isoform with a pI value corresponding to q-IV in PMT-treated
cells (Fig. 1G; Table 1).

We excluded the possibility that the Gα11 antibody could react
with Gαq,-1 by testing the ability of the Gαq,-1 and Gα11 antibodies to

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References:

1. G-Proteins and the Pasteurella multocida Toxin

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no discernable effect on the pl or molecular mass of the Gq subunits (Fig. S1C; Table S1).

PMT stimulates the stable covalent modification of G-proteins

It was important to establish whether the additional isoforms detected in PMT-treated cells arose as a consequence of normal activation induced by PMT or if they were directly PMT-modified. Cells were challenged with the neuropeptide bombesin, which acts through a Gq-coupled receptor to stimulate phospholipase C (PLC) activation culminating in the release of Ca2+ from intracellular stores [32]. Bombesin at a concentration of 30 nM significantly enhanced GTP stores [32]. Bombesin at a concentration of 30 nM enhanced GTP binding to Gq, although it does not activate Gq downstream of the activation induced by PMT or if they were directly PMT-induced (Fig. 4B, C). This suggested that Gq-coupled receptor activation did not stimulate the stable covalent modification of Gq.

We have previously demonstrated that PMT induced the phosphorylation of Gq, on Tyr369 [9]. We stimulated membrane fractions with bombesin in the presence of sodium vanadate, a potent tyrosine phosphatase inhibitor, in order to prevent the dephosphorylation of Gq11. Bombesin activation of Gq11 in the membrane fractions was confirmed by the trypsin protection assay. Bombesin significantly enhanced GTPS binding to Gq11 (p = 0.002), by up to 50% in some cases, the most likely explanation being that its action accelerated the rate of nucleotide exchange (Fig. 4D). The additional isoforms detected in membranes stimulated with bombesin appeared to be identical to those found in PMT-treated cells. However, the additional Gq11 and Gq isoforms were also found in membranes derived from unstimulated cells that had been treated with sodium vanadate alone (Fig. 4E, F and H). These findings suggest that PMT modification of Gq11 and Gq produces a similar pl shift as the tyrosine phosphorylation of these Gq subunits.

The appearance of the additional isoforms observed in PMT-treated cells could not be blocked by the competitive kinase inhibitors Su6656 or Su638, although these inhibitors were effective at blocking pervanadate-induced phosphorylation of focal adhesion kinase (FAK) (Fig. S2). We have previously shown that a mutant PMT (PMT<sub>631/658</sub>) can stimulate the tyrosine phosphorylation of Gq, although it does not activate Gq downstream of the activation induced by PMT or if they were directly PMT-induced (Fig. S3). Treatment of Swiss 3T3 cells with PMT<sub>631/658</sub> did not result in the covalent modification of Gq11 or Gq (Fig. S3). Moreover, tyrosine phosphorylation is a transient reversible modification that cannot be readily detected unless tyrosine phosphatases are inhibited. The PMT-induced modification of Gq was detected in the absence of sodium vanadate, indicating that the PMT-induced modification was covalent and stable.

Prolonged treatment of cells with PMT has differential effects on G-proteins

PMT treatment decreased the abundance of some of the pre-existing Gq11 and Gq isoforms in membrane fractions. To explore if PMT caused G-protein removal from membranes, Swiss 3T3 cells were treated with PMT at a concentration of 1 nM for 16 h. This treatment did not cause loss of Gq11 from the membrane (Fig. 5A), but resulted in the complete loss of the most basic isoforms of Gq and Gq11, q-III and q-VI, respectively (Fig. 5B, C). These isoforms were also found in membranes derived from unstimulated cells, that had been treated with sodium vanadate alone (Fig. 5G). This suggests that Gq11 isoforms reflects a modification that interferes with the Gq interaction with the antigen recognition site, which is at the C-terminus of Gq, as the loss of Gq from membranes could also be demonstrated with an antiserum against an internal epitope of Gq3 (Fig. 5F). Cytoplasmic extracts of cells that had received prolonged treatment with PMT were probed with anti-Gq11, q-III and q-VI did not undergo an evident change in abundance. We speculate that the loss of detection of Gq11 isoforms q-III and q-VI is a result of the covalent modification of these isoforms to q-I and q-IV, respectively, induced by PMT.

In contrast, prolonged treatment of Swiss 3T3 cells with PMT generally resulted in the almost complete loss of Gq from membranes (Fig. 5E, F). It is unlikely that the failure to detect the Gq isoforms reflects a modification that interferes with the Gq3, q-III antigen recognition site, which is at the C-terminus of Gq, as the loss of Gq from membranes could also be demonstrated with an antiserum against an internal epitope of Gq3 (Fig. 5F). Cytoplasmic extracts of cells that had received prolonged treatment with PMT were probed with anti-Gq11, q-III antibody, but no increase in Gq subunits could be detected in these fractions (Fig. 5G). It appears that the sequential loss of Gq from membranes proceeds by covalent modification of Gq isoforms i-I and i-IV to produce isoforms i-I and i-III, respectively, followed over time by the loss of isoforms i-I and i-III from the membranes (Fig. 5H-J). In some cases only partial loss of Gq isoforms was observed over this period (data not shown).

PMT inhibits the GTPase activity of Gq

PMT did not significantly enhance GTPS binding to Gq11 in contrast to bombesin (data not shown). Due to its enzymatic nature, PMT required a longer incubation time to promote GTP binding to Gq compared to bombesin [9]. Therefore, it is likely that during the course of the incubation, Gq was gradually saturated by GTPS, thereby preventing the detection of PMT-enhanced GTPS binding to Gq above background levels. When GTP was used instead of GTPS, PMT significantly enhanced GTP binding to Gq as measured by trypsin protection (p = 0.03), by up to 30% (Fig. 6A), in contrast to bombesin (Fig. 6B). This

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**Table 1. Analysis of pl values of Gq family isoforms after treatment with PMT.**

| Isoform | Control (pl) | PMT-treated (pl) |
|---------|--------------|------------------|
|         | Gq<sub>q/11</sub> | Gq<sub>q</sub> | Gq<sub>q/11</sub> | Gq<sub>q</sub> | Gq<sub>q/11</sub> |
| q-I     | -            | -                | 5.39±0.04 | 5.42±0.02 | -            |
| q-II    | 5.45±0.04    | 5.49±0.02        | 5.49±0.01 | 5.51±0.02 | -            |
| q-III   | 5.61±0.05    | 5.59±0.05        | 5.60±0.08 | 5.60±0.03 | -            |
| q-IV    | -            | -                | 5.64±0.01 | -         | 5.64±0.02 |
| q-V     | 5.76±0.09    | 5.75±0.01        | 5.76±0.09 | -         | 5.73±0.01 |
| q-VI    | 5.89±0.1     | 5.85±0.01        | 5.83±0.02 | -         | 5.82±0.05 |

The samples were as described in the legend to Figure 1 and the results are expressed as the mean ± standard error of the mean (n = 3).

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finding suggested that PMT might inhibit the GTPase activity of Gαq, to prevent the hydrolysis of GTP to GDP.

Bombesin stimulated the steady-state GTPase activity in Swiss 3T3 membrane preparations by up to 30%, whereas pretreatment of cells with PMT at 150 pM for 4 h reduced the basal and bombesin-stimulated GTPase activity in membrane preparations (Fig. 6C). To further decrease the basal steady-state GTPase level, cells were pre-treated with cholera toxin, which ADP-ribosylates Gαs to inhibit its GTPase activity. Cholera toxin caused an increase in the molecular weight of both the long and short forms of Gαq, due to the addition of ADP ribose (Fig. 6D). Pre-treatment of cells with both cholera toxin and PMT further decreased the basal GTPase activity in membrane preparations, compared to cells pre-treated with PMT alone. Bombesin stimulated the steady state GTPase activity by up to 50% in cells pre-treated with cholera toxin, whereas the additional pre-treatment of cells with PMT reduced the bombesin-stimulated

Figure 2. PMT induces the covalent modification of Gαi. (A) Membrane proteins from Swiss 3T3 cells were separated by SDS PAGE and Western blotted with anti-Gαi-1, anti-Gαi-1-2 or anti-Gαi-1-3 antibody, as indicated. (B) A recombinant Gαi subunit was analysed by SDS PAGE and Western blotted with anti-Gαi-1, Gαi-1-2 or Gαi-1-3 antibody, as indicated. Membrane proteins from Swiss 3T3 cells (C, E, G) left untreated or (D, F, H) treated with 150 pM PMT for 4 h were separated by 2-D gel electrophoresis and Western blotted with (C, D) anti-Gαi-1, (E, F) anti-Gαi-1-2 or (G, H) anti-Gαi-1-3 antibody. Samples from at least 3 independent experiments were resolved with similar results.

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GTase activity in membrane preparations, indicating that PMT inhibits the GTase activity of Gαi but not Gs (Fig. 6C).

Discussion

PMT executes its cellular effects through the activation of the heterotrimeric G-proteins, Gq, G12 and Gi [8–13]. This has been shown to occur in recombinant Gi by PMT-induced deamidation of Gln-205 to glutamic acid, which inhibits its intrinsic GTase activity [14]. This work here reports complements these studies by investigating covalent modifications of G-proteins in Swiss 3T3 cells treated with PMT. PMT treatment consistently led to the appearance of new isoforms at a lower pI for both Gαq and Gα11. PMT also stimulated the covalent modification of members of the Gi family. The Gα2 family proteins, unlike the other G-protein families, have predicted pI values within the alkaline pH range (>pH 8) and such proteins are difficult to resolve by 2-D gel electrophoresis [33]. Gα13, but not Gα12, subunits displayed a reproducible pattern and PMT treatment led to new Gα13 isoforms at slightly higher pI values. We found no evidence that PMT stimulates the covalent modification of Gαs, although the glutamine residue targeted by PMT is conserved in all G-proteins.

Stimulation of Gq-coupled receptors by bombesin only resulted in the detection of the additional Gαq/11 isoforms observed in PMT-treated cells when vanadate was present. The addition of sodium vanadate per se led to a similar pattern of isoforms to those observed in PMT-treated cells. However it is likely that these different treatments lead to different modifications. The modification of Gαq/11 and Gα13 stimulated by PMT was detected without sodium vanadate, and is thus indicative of a stable covalent modification such as deamidation, whereas tyrosine phosphorylation is a transient covalent modification. We previously showed that a src family kinase mediates the phosphorylation of Gαq in response to PMT [9]. However, pre-treatment of cells with a specific src kinase family inhibitor, SU6656, or a broad spectrum kinase inhibitor, S6638, did not prevent PMT from stimulating the covalent modification of Gαq and Gα13, despite each kinase inhibitor being effective at blocking FAK phosphorylation. It is possible that the kinase inhibitors failed to completely block PMT-stimulated phosphorylation of G-proteins, due to their competitive nature and the enzymatic nature of PMT. However, this would suggest that deamidation by PMT results in the stable phosphorylation of these Gα subunits that is not reversed by the action of phosphatases, which is unlikely.

Deamidation and tyrosine phosphorylation of a Gα subunit would have a similar effect on the isoelectric point. The PMT-induced deamidation of in-vitro translated Gαq and recombinant Gαq,2 was reported to cause an acidic pI shift of 0.05 and 0.07, respectively [14]. This compares with the acidic pI shift of approximately ~0.15 for both Gαq and Gα13 that we have observed. There are various possible interpretations of this apparent discrepancy. First, pI shifts are known to be variable and depend on the overall pI of a protein and its local context [34], and thus Gα13 expressed in E. coli may behave differently because of the absence of post-translational modifications. Alternatively, the PMT-induced modification in cells may differ from that observed following expression in E. coli.

PMT is reported not to activate Gi13, as PMT could not induce the activation of PLC in Gq-deficient cells [12], and further analysis using Gαq/Gα13 chimeras also confirmed that PMT did not.

Table 2. Analysis of pI values of Gαq family isoforms after treatment with PMT.

| Isoform     | Control (pI) | PMT-treated (pI) |
|-------------|--------------|------------------|
|             | Gαq,1        | Gαq,1,2          | Gαq,1,2,3        |
| Gαq,1       | 5.11±0.01    | 5.09±0.03        | 5.10±0.01        |
| Gαq,1,2     | -            | 5.22±0.03        | 5.18±0.03        |
| Gαq,1,2,3   | -            | -                | 5.17±0.01        |

The samples were as described in the legend to Figure 2 and the results are expressed as the mean ± standard error of the mean (n=3).

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Table 3. Analysis of pI values of Gα13 isoforms after treatment with PMT.

| Isoform | Control (pI) | PMT-treated (pI) |
|---------|--------------|------------------|
| 13-I    | 8.15±0.02    | 8.15±0.07        |
| 13-II   | -            | 8.24±0.0          |
| 13-III  | 8.54±0.02    | 8.54±0.02        |
| 13-IV   | 8.90±0.05    | 8.90±0.01        |
| 13-V    | -            | 9.04±0.01        |

The samples were as described in the legend to Figure 4 and the results are expressed as the mean ± standard error of the mean (n=3).

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not lead to $G_{11}$-linked stimulation of PLC [35]. We were therefore surprised that PMT stimulated the covalent modification of $G_{11}$.

$G_{aq}$ and $G_{11}$ each contain Gln-209 that is functionally equivalent to Gln-205 in $G_{ai2}$ and it would be unlikely that $G_{11}$ could be deamidated and yet not activated by PMT, as the loss of the functional Gln would affect the GTPase activity of the G-protein. While this manuscript was in preparation, Kamitani et al. published evidence that an antibody against deamidated $G_{aq}$ subunits recognised $G_{11}$ in PMT-treated mouse embryonic fibroblasts [36]. This result provides further evidence that $G_{11}$ is also a substrate for PMT. In their experiments there was a small stimulation of PLC in cells expressing $G_{11}$. All the other papers addressing this issue have used the same source of $G_{aq/11}$-deficient MEF cells, whereas our work uses Swiss 3T3 cells. Further investigation of these puzzling and partially contradictory results is required.

PMT treatment of cells led to new $G_{13}$ isoforms at slightly higher (0.09–0.15) pI values. The PMT catalytic triad has high structural similarity to eukaryotic transglutaminases [14], and it is possible that PMT can also function as a transglutaminase, in a similar manner to the cytotoxic necrotizing factor (CNF) which was originally considered to be a deamidase, but was later found to cause transglutamination in cells [37]. Transglutaminases catalyse the acyl transfer between the γ-carboxamido of a peptide bound glutamine (acyl donor) to a primary amine (acyl acceptor). When water functions as an acyl acceptor the result is glutamine deamidation [38]. The choice between deamidation and transglutamination is influenced by the environment of the targeted glutamine residue [39,40]. As transglutamination would impart a

**Figure 4. Sodium vanadate treatment mimics PMT effects on $G_{aq}$ and $G_{11}$**

(A) Indo-1 AM labelled Swiss 3T3 cells were treated with 30 nM bombesin for 10 s (marked with solid bar beneath trace) and intracellular Ca$^{2+}$ release was measured. Membrane proteins from Swiss 3T3 cells that were either (B) untreated or (C) treated with 30 nM bombesin for 1 min were separated by 2-D gel electrophoresis and Western blotted with anti-$G_{aq/11}$ antibody. (D) Swiss 3T3 membrane proteins were incubated in the presence or absence of 30 nM bombesin for 20 min with or without 0.05 nM GTPγS. The proteins were then analysed for trypsin protection as described under Materials and Methods, and activated $G_{aq/11}$ was separated by SDS PAGE and Western blotted with anti-$G_{aq/11}$ antibody. Quantification of activated $G_{aq/11}$ (lower panel) was determined by densitometric scanning and these data were analysed using factorial analysis of variance (ANOVA). The induction of activation shown is relative to the density of the band without GTPγS or bombesin. Bombesin significantly enhanced GTPγS binding to $G_{aq/11}$ (* p = 0.002). Membrane proteins from Swiss 3T3 cells were incubated with (E) 1 mM sodium vanadate for 20 min at 37°C or (F) 1 mM sodium vanadate and 30 nM bombesin for 20 min at 37°C, proteins were separated by 2-D gel electrophoresis and Western blotted with anti-$G_{aq/11}$ antibody. Membrane proteins from Swiss 3T3 cells were incubated (G) without or (H) with 1 mM sodium vanadate for 20 min at 37°C, proteins were separated by 2-D gel electrophoresis and Western blotted with anti-$G_{aq/11}$ antibody. Samples from at least 3 independent membrane preparations were resolved with similar results. doi:10.1371/journal.pone.0047188.g004
positive charge to produce an alkaline shift, it is possible that PMT preferentially transglutamates Gα₁₃ in cells.

The removal of G-proteins from the membrane is a regulatory phenomenon that can follow prolonged G-protein activation [41]. The ADP-ribosylation of Gs by cholera toxin leads to its downregulation, although ADP-ribosylation of Gα_i by pertussis toxin does not result in its degradation [42]. We observed that prolonged treatment of cells with PMT caused the loss of Gα_i but not Gα_q from membranes prepared from Swiss 3T3 cells. Furthermore Gα_i could not be detected in the cytoplasm following prolonged PMT treatment. Orth et al. had suggested that overnight treatment of Swiss 3T3 cells with 1 nM PMT uncoupled Gα_i from its receptor, as the Gα_i-linked agonist lysophosphatidic acid could not stimulate GTPγS binding to Gα_i in membranes derived from these cells [10]. The loss of Gα_i from the membrane that we observed over this time period would provide a more likely explanation for their observation. Furthermore, the site of the PMT-induced modification, Gln-205, is not thought to be linked to receptor interaction. A similar differential degradation has been observed with Rho proteins following modification by CNF [43].

We found that PMT could promote the binding of GTP to Gα_q/11, whereas bombesin could not, which suggested that the action of PMT inhibits the GTPase activity of Gα_q/11. PMT significantly inhibited the bombesin-mediated stimulation of steady-state GTPase activity in Swiss 3T3 membrane preparations. These results complement the demonstration that PMT inhibits the GTPase activity of E. coli-expressed Gα_i [10,14]. Furthermore, pre-treatment of cells with cholera toxin and PMT resulted in a greater inhibition of GTPase activity, supporting the view that PMT does not affect Gs.

In conclusion, our results demonstrate that treatment of Swiss 3T3 cells with PMT induces the irreversible modification of G-proteins belonging to the Gi and Gq families resulting in an acidic pI shift, which is consistent with the observation that PMT catalyses deamidation of recombinantly expressed Gi causing a similar shift in pI. We found that PMT treatment causes an alkaline pI shift in Gα₁₃ and speculate that PMT might preferentially transglutaminate Gα₁₃. Working with cells enables the PMT/G-protein interaction to be investigated in a more natural context than when working with recombinantly expressed proteins. However, the further interpretation of results is impeded by the near impossibility of purifying these low abundance proteins in a modified form from cell lines, and thus both in-vitro and in-vivo studies are required to unravel the complexity of the toxin/G-protein interactions.

Figure 5. Prolonged exposure of Swiss 3T3 cells to PMT causes the loss of Gα_i but not Gα_q from cell membranes. (A) Membrane proteins from Swiss 3T3 cells left untreated (lane 1) or treated with PMT at 1 nM for 16 h (lane 2) were separated by SDS PAGE and Western blotted with anti-Gα_q/11 antibody. Membrane proteins from Swiss 3T3 cells (B) left untreated, or treated with 1 nM PMT for (C) 4 h or (D) 16 h were separated by 2-D gel electrophoresis and Western blotted with anti-Gα_q/11 antibody. Samples from at least 3 independent experiments were resolved with similar results. Membrane proteins from Swiss 3T3 cells left untreated (lane 1) or treated with 1 nM PMT for 16 h (lane 2) were separated by SDS PAGE and Western blotted with (E) anti-Gα_q/3 antibody or (F) an antibody recognising an internal epitope of Gα_q/2. (G) Cytoplasmic proteins from Swiss 3T3 cells left untreated (lane 1) or treated with 1 nM PMT for 16 h (lane 2) were separated by SDS PAGE and Western blotted with anti-Gα_q/3 antibody. Membrane proteins from Swiss 3T3 cells (H) left untreated, treated with 1 nM PMT for (I) 4 h or (J) 16 h were separated by 2-D PAGE and Western blotted with anti-Gα_q/3 antibody. Samples from 3 independent experiments were resolved with similar results.

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Figure S1 PMT does not induce the covalent modification of Gα. (A) Membrane proteins from Swiss 3T3 cells were separated by SDS PAGE and Western blotted with anti-Gα antibody. Membrane proteins from Swiss 3T3 cells left (B) untreated or (C) treated with 150 pM PMT for 4 h were separated by 2-D gel electrophoresis and Western blotted with anti-Gα antibody. Samples from at least 3 independent experiments were resolved with similar results.

Supporting Information

Figure S1  PMT does not induce the covalent modification of Gα. (A) Membrane proteins from Swiss 3T3 cells were separated by SDS PAGE and Western blotted with anti-Gα antibody. Membrane proteins from Swiss 3T3 cells left (B) untreated or (C) treated with 150 pM PMT for 4 h were separated by 2-D gel electrophoresis and Western blotted with anti-Gα antibody. Samples from at least 3 independent experiments were resolved with similar results.

Figure S2 Kinase inhibitors do not block PMT induced modification of Gαq/11 or Gαi. Swiss 3T3 cells were either not treated (Lane 1) or pre-treated (Lane 2) for 1 h with (A) SU6656 or (B) St638, then stimulated with 0.5 nM pervanadate for 5 min. The cells were lysed in SDS-buffer and proteins were resolved by SDS PAGE followed by Western blotted with an anti-phospho-FAK antibody. Three independent experiments gave similar results. Swiss 3T3 cells were (C, D, G, H) not treated or pre-treated with either (E, I) SU6656 or (F, J) St638 and then either treated with (D, E, F, H, I, J) 150 pM PMT or (C, G) not treated with PMT. Samples were resolved from 3 independent experiments with similar results. Membrane proteins were separated by 2-D gel electrophoresis and Western blotted with anti-Gαq/11 antibody or (G-J) anti-Gαi-1-3 antibody. Samples were resolved from 2 independent experiments with similar results.

Figure S3 Mutant PMT does not induces the covalent modifications of Gαq or Gαi. Membrane proteins from Swiss 3T3 cells (A, C) left untreated or (B, D) treated with 150 pM PMT for 4 h, separated by 2-D gel electrophoresis and Western blotted with either (A, B) anti-Gαq/11 or (C, D) anti-Gαi-1-3 antibodies. Samples from 3 independent experiments were resolved with similar results.

Table S1 Analysis of pI values of Gα family isoforms after treatment with PMT. The samples were as described in

Supporting Information

Figure S1  PMT does not induce the covalent modification of Gα. (A) Membrane proteins from Swiss 3T3 cells were separated by SDS PAGE and Western blotted with anti-Gα antibody. Membrane proteins from Swiss 3T3 cells left (B) untreated or (C) treated with 150 pM PMT for 4 h were separated by 2-D gel electrophoresis and Western blotted with anti-Gα antibody. Samples from at least 3 independent experiments were resolved with similar results.

(TIF)
the legend to Figure. S1 and the results are expressed as the mean ± standard error of the mean.

(DOC)

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