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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, G_q, G_{12} and G_i. PMT has been shown by others to lead to the deamidation of recombinant $G\alpha_i$ at Gln-205 to inhibit its intrinsic GTPase activity. We have investigated modification of native $G\alpha$ 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 $G\alpha$ subunit of the G_q and G_i families following PMT treatment of Swiss 3T3 cells, which is consistent with the deamidation of these $G\alpha$ subunits. Surprisingly, PMT also induced a similar modification of $G\alpha_{11}$, a member of the G_q family of G-proteins that is not activated by PMT. Furthermore, an alkaline change in the isoelectric point of $G\alpha_{13}$ was observed following PMT treatment of cells, suggesting differential modification of this $G\alpha$ subunit by PMT. G_s was not affected by PMT treatment. Prolonged treatment with PMT led to a reduction in membrane-associated $G\alpha_i$, but not $G\alpha_q$. We also show that PMT inhibits the GTPase activity of G_q.

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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 (G_q, G_{12}, G_i and G_s) according to their constituent alpha subunit, which is a guanine nucleotide binding protein that can exist in an inactive GDP-bound or an active GTP-bound form [2]. Activation of a GPCR causes a conformational change in its cognate $G\alpha$ 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 $G\alpha$ 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 G_s and G_i families activate or inhibit adenylate cyclase, respectively [4]. The G_q family activates phospholipase C (PLC) [5], while the G_{12} 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 G_q, G_{12} and G_i families [8–13]. PMT catalyses the deamidation of recombinant G_i 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-^{32}P) GTP was obtained from PerkinElmer LAS. Anti-$G\alpha_{q/11}$ (sc-392), anti-$G\alpha_{11}$ (sc-394), anti-$G\alpha_s$ (sc-387), anti-$G\alpha_{13}$ (sc-410) and anti-$G\alpha_{i\text{-}2}$ (internal: sc-7276) antibodies were from Santa Cruz Biotechnology. Anti-$G\alpha_q$ (371752), anti-$G\alpha_{i\text{-}1}$ (371720), anti-$G\alpha_{i\text{-}1\text{-}2}$ (371723) and anti-$G\alpha_{i\text{-}1\text{-}3}$ (371729: which is known to cross react with $G\alpha_{i\text{-}1}$ and $G\alpha_{i\text{-}2}$) antibodies were purchased from Calbiochem-Novabiochem. Phospho-FAK (Tyr^{397}) 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 $G\alpha_q$ subunit (371765) was purchased from Calbiochem-Novabiochem. Recombinant His6-tagged human $G\alpha_{i\text{-}1}$ was expressed and purified from *E. coli* containing pProEX-HTb, which was provided as a kind gift by Professor David Siderovski (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 St638 (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 proteinase inhibitors (Complete^{TM}, 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 $MgCl_2$, 0.1 mM EDTA, pH 7.4, containing proteinase 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 a fresh tube, snap frozen in liquid nitrogen and stored at −70°C. The pellet was washed and suspended in 10 ml of membrane buffer. After a second centrifugation step the membrane pellet was suspended in membrane buffer to a protein concentration of 1 mg/ml and stored at −70°C.

SDS PAGE and urea gel electrophoresis

Membrane proteins were resolved by SDS PAGE on 12.8% acrylamide/0.06% bis acrylamide gels, or on these same gels containing 6M urea to separate the closely migrating $G\alpha_{11}$ and $G\alpha_q$ subunits as described [18]. Proteins were transferred to PVDF membranes and immunoblotted as described below.

2-D gel electrophoresis

Swiss 3T3 membrane proteins were resolved by 2-D gel electrophoresis, as described [19]. The immunodetection of Gα subunits was performed by incubating the membrane overnight at 4°C with primary antibody at a dilution of 1:1000, followed by incubation with horseradish peroxidase-coupled secondary antibody at a dilution of 1:10000 (SouthernBiotech) for 1 h at room temperature. The membrane was incubated with ECL^{TM} chemiluminescent substrate (GE HealthCare) and signals were detected using an automatic X-Ray film processor (Jungwon Precision Industries Co.).

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 488 nm. The emission ratio (R) was converted to a calcium concentration after calibration (see reference 20] in which [Ca]i (nM) = 1028(R-0.86)/(12-R) and autofluorescence was less that 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 18,000×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 $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1% $C_{12}E_{10}$ (polyoxyethylene 10-lauryl ether), 0.1 mM phenylmethylsulfonyl fluoride), vortexed, incubated on ice for 20 min and centrifuged at 18,000×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 mg/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 $G\alpha_{q/11}$. The induction of trypsin protection by GTPγS and GTP alone or in the presence of bombesin or PMT were quantified relative to untrypsinised G_q using scanning densitometry (GeneTools, 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 tryptic digestion; however tryptic 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].

Measurement of high-affinity GTPase activity

Determination of GTPase activity was essentially as described [23]. High-affinity GTPase activity was determined by subtraction of P_i release in membranes incubated with 50 μM of GTP (low-affinity GTPase activity) from that with 0.5 μM GTP (total GTPase activity).

Results

PMT stimulates an acidic modification of $G\alpha_q$ and $G\alpha_i$ family proteins

$G\alpha_{q/11}$ antiserum detected both $G\alpha_q$ and $G\alpha_{11}$ 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 $G\alpha_q$ (which aberrantly runs slower in this system than G_{11} [24]) was more abundantly expressed than $G\alpha_{11}$ in these cells (Fig. 1A). A similar relative abundance has been shown in rat neurons [25]. Four distinct $G\alpha_{q/11}$ molecular isoforms, designated q-II, q-III, q-V and q-VI, were resolved by 2-D gel electrophoresis followed by immunoblotting with anti-$G_{q/11}$ antibody in membranes derived from untreated cells (Fig. 1B). These plus two additional isoforms, q-I and q-IV, were detected by 2D PAGE and Western blot analysis of membrane fractions derived from cells treated with PMT (150 pM) for 4 h (Fig. 1C; Table 1).

Antiserum directed only against $G\alpha_q$ detected two isoforms with pI values corresponding to q-II and q-III (Fig. 1D; Table 1) in untreated cells. The $G\alpha_q$ antiserum detected an additional isoform with a pI value corresponding to q-I in PMT-treated cells (Fig. 1E; Table 1). Antiserum directed only against $G\alpha_{11}$ detected two

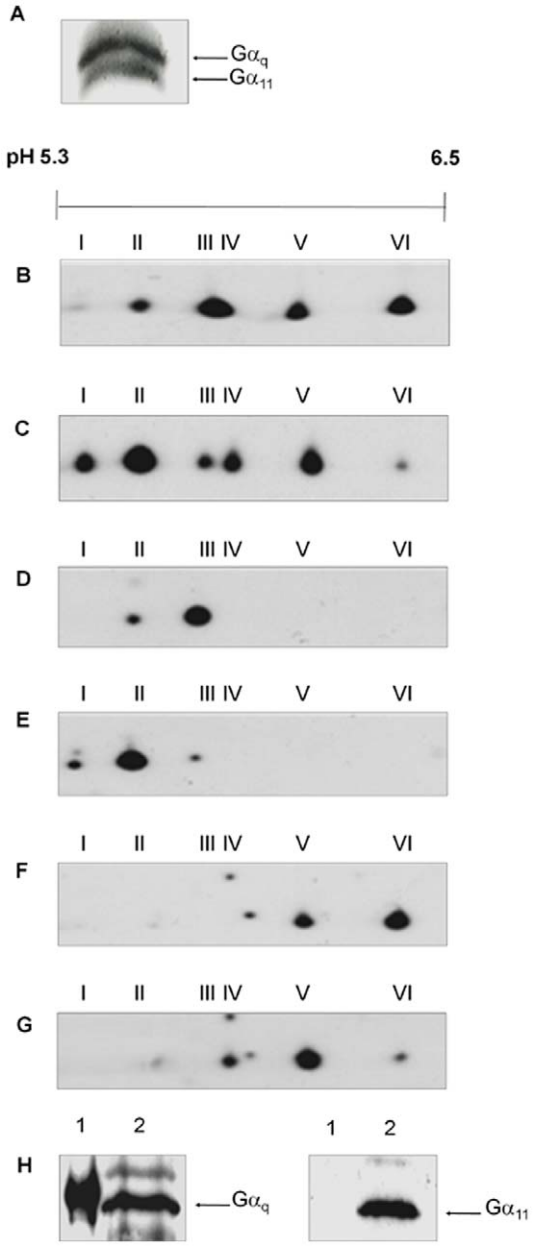

Figure 1. PMT induces the covalent modification of $G\alpha_q$ and $G\alpha_{11}$. (**A**) Membrane proteins from Swiss 3T3 cells were separated by urea gel electrophoresis and Western blotted with anti-$G\alpha_{q/11}$ antibody. The locations of $G\alpha_q$ and $G\alpha_{11}$ are indicated by arrows. Membrane proteins from Swiss 3T3 cells (**B**, **D**, **F**) left untreated or (**C**, **E**, **G**) treated with 150 pM PMT for 4 h were separated by 2-D gel electrophoresis and Western blotted with (**B**, **C**)anti-$G\alpha_{q/11}$, (**D**, **E**)anti-$G\alpha_q$ or (**F**, **G**) anti-$G\alpha_{11}$ antibody. (**H**) Recombinant $G\alpha_q$ subunit (lane 1) and membrane proteins from Swiss 3T3 cells (lane 2) were separated by SDS PAGE and Western blotted with anti-$G\alpha_q$ (left panel) or -$G\alpha_{11}$ (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 $G\alpha_{11}$ antibody could react with $G\alpha_q$ by testing the ability of the $G\alpha_q$ and $G\alpha_{11}$ antibodies to react with a recombinant $G\alpha_q$ subunit. The $G\alpha_q$ but not the $G\alpha_{11}$ antiserum could detect the $G\alpha_q$ subunit (Fig. 1H). The experimentally determined pI values for the $G\alpha_{q/11}$ isoforms (Table 1) are similar to the predicted pI values of 5.48 and 5.70 for murine $G\alpha_q$ and $G\alpha_{11}$, respectively [26,27].

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

$G\alpha_{i\text{-}1}$ 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 immunoblotting (Fig. 2C, D; Table 2). The $G\alpha_{i\text{-}1\text{-}2}$ antiserum detected two $G\alpha_i$ 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 $G\alpha_{i\text{-}1\text{-}3}$ antiserum detected 3 $G\alpha_i$ 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 $G\alpha_{i\text{-}1\text{-}3}$ antiserum also detected these and one additional isoform, i-III in PMT-treated cells (Fig. 2H; Table 2).

The predicted pI values of murine $G\alpha_{i\text{-}1}$, $G\alpha_{i\text{-}2}$ and $G\alpha_{i\text{-}3}$ are 5.69, 5.28 and 5.50, respectively [29]. It seems probable that isoforms i-I and i-II detected by the $G\alpha_{i\text{-}1\text{-}2}$ antiserum belong to the $G\alpha_{i\text{-}2}$ subclass, as isoforms of the $G\alpha_{i\text{-}1}$ subclass are expected to have a more basic pI, and $G\alpha_{i\text{-}1}$ was not detected in Swiss 3T3 cells. Isoforms i-III and i-IV are therefore likely to belong to the $G\alpha_{i\text{-}3}$ subclass. Orth *et al.* resolved $G\alpha_{i\text{-}1}$ and $G\alpha_{i\text{-}2}$ 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 $G\alpha_{i\text{-}2}$ [14]. Our results suggest that PMT catalyses the acidic covalent modification of $G\alpha_{i\text{-}2}$ and $G\alpha_{i\text{-}3}$.

PMT induces an alkaline modification of $G\alpha_{13}$

The two members of the $G\alpha_{12}$ family, $G\alpha_{12}$ and $G\alpha_{13}$, are ubiquitously expressed [30]. $G\alpha_{13}$ was detected in Swiss 3T3 membranes using antiserum against $G\alpha_{13}$ (Fig. 3A). Three $G\alpha_{13}$ 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 $G\alpha_{13}$ isoforms seem to be the result of an alkaline pH shift, in contrast to the effect of PMT on $G\alpha_{q/11}$ and $G\alpha_i$ isoforms. Under our experimental conditions, $G\alpha_{12}$ could not be resolved by 2-D gel electrophoresis.

PMT does not induce any modification of $G\alpha_s$

The alpha subunits of the ubiquitously expressed G_s 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 $G\alpha_s$, with $G\alpha_s$-large being more abundantly expressed than $G\alpha_s$-small (Fig. S1A). Six isoforms of $G\alpha_s$-large (s-I to s-VI) and two isoforms of $G\alpha_s$-small (s-VII and s-VIII) were resolved in membranes derived from Swiss 3T3 cells by 2-D gel electrophoresis, followed by immunoblotting with the $G\alpha_{s/olf}$ antiserum (Fig. S1B; Table S1). The $G\alpha_s$-large isoforms were detected at a more acidic pI than the $G\alpha_s$-small isoforms, which concurs with previous findings [19]. PMT showed

Table 1. Analysis of pI values of $G\alpha_q$ family isoforms after treatment with PMT.

	Control (pI)			PMT-treated (pI)		
Isoform	**$G\alpha_{q/11}$**	**$G\alpha_q$**	**$G\alpha_{11}$**	**$G\alpha_{q/11}$**	**$G\alpha_q$**	**$G\alpha_{11}$**
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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no discernable effect on the pI or molecular mass of the $G\alpha_s$ 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 G_q-coupled receptor to stimulate phospholipase C (PLC) activation culminating in the release of Ca^{2+} from intracellular stores [32]. Bombesin at a concentration of 30 nM effectively stimulated Ca^{2+} release from cells (Fig. 4A), but no additional $G\alpha_{q/11}$ isoforms were detected by 2D PAGE and Western blot analysis of a membrane fraction derived from cells exposed to bombesin (Fig. 4B, C). This suggested that $G\alpha_q$-coupled receptor activation did not stimulate the stable covalent modification of $G\alpha_q$.

We have previously demonstrated that PMT induced the phosphorylation of $G\alpha_q$ 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 $G\alpha_{q/11}$. Bombesin activation of $G\alpha_{q/11}$ in the membrane fractions was confirmed by the trypsin protection assay. Bombesin significantly enhanced GTPγS binding to $G\alpha_{q/11}$ ($p = 0.002$), 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 $G\alpha_{q/11}$ and $G\alpha_i$ 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 $G\alpha_{q/11}$ and $G\alpha_i$ produces a similar pI shift as the tyrosine phosphorylation of these Gα subunits.

The appearance of the additional isoforms observed in PMT-treated cells could not be blocked by the competitive kinase inhibitors Su6656 or St638, 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^{C1165S}) can stimulate the tyrosine phosphorylation of G_q, although it does not activate G_q downstream signalling [9]. Treatment of Swiss 3T3 cells with PMT^{C1165S} did not result in the covalent modification of $G\alpha_q$ or $G\alpha_i$ (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 Gα subunits 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 $G\alpha_{q/11}$ and $G\alpha_i$ 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 $G\alpha_{q/11}$ from the membrane (Fig. 5A), but resulted in the complete loss of the most basic isoforms of $G\alpha_q$ and $G\alpha_{11}$, q-III and q-VI, respectively (Fig. 5B, C, and D), while isoforms q-II and q-IV did not undergo an evident change in abundance. We speculate that the loss of detection of $G\alpha_{q/11}$ 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 $G\alpha_i$ from membranes (Fig. 5E, F). It is unlikely that the failure to detect the $G\alpha_i$ isoforms reflects a modification that interferes with the $G\alpha_{i\text{-}1\text{-}3}$ antigen recognition site, which is at the C-terminus of $G\alpha_i$, as the loss of $G\alpha_i$ from membranes could also be demonstrated with an antiserum against an internal epitope of $G\alpha_{i\text{-}2}$ (Fig. 5F). Cytoplasmic extracts of cells that had received prolonged treatment with PMT were probed with anti-$G\alpha_{i\text{-}1\text{-}3}$ antibody, but no increase in $G\alpha_i$ subunits could be detected in these fractions (Fig. 5G). It appears that the sequential loss of $G\alpha_i$ from membranes proceeds by covalent modification of $G\alpha_i$ isoforms i-II 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 $G\alpha_i$ isoforms was observed over this time period (data not shown).

PMT inhibits the GTPase activity of G_q

PMT did not significantly enhance GTPγS binding to $G\alpha_{q/11}$ in contrast to bombesin (data not shown). Due to its enzymatic nature, PMT required a longer incubation time to promote GTP binding to $G\alpha_q$ compared to bombesin [9]. Therefore, it is likely that during the course of the incubation, $G\alpha_q$ was gradually saturated by GTPγS, thereby preventing the detection of PMT-enhanced GTPγS binding to $G\alpha_q$ above background levels. When GTP was used instead of GTPγS, PMT significantly enhanced GTP binding to $G\alpha_q$ as measured by trypsin protection ($p = 0.03$), by up to 30% (Fig. 6A), in contrast to bombesin (Fig. 6B). This

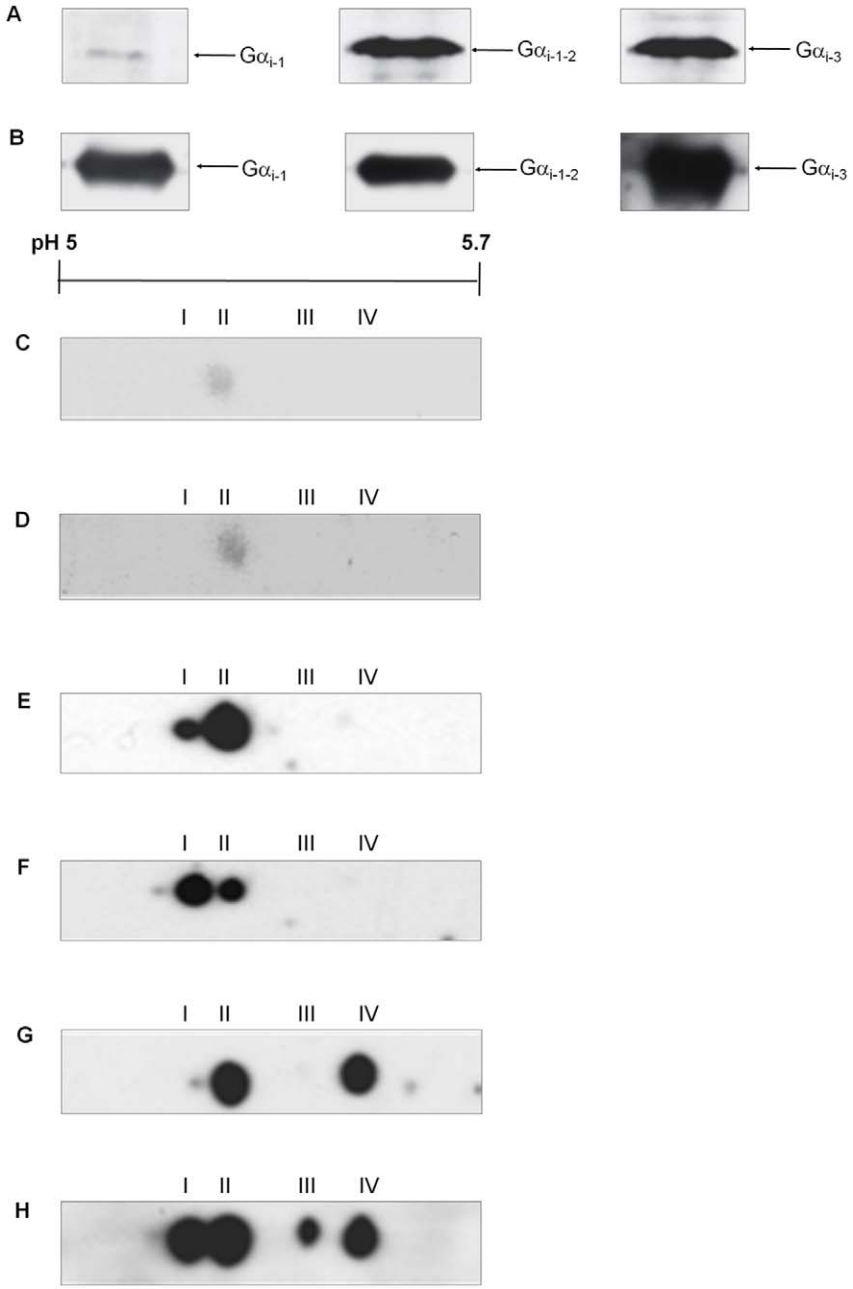

Figure 2. PMT induces the covalent modification of $G\alpha_i$. (**A**) Membrane proteins from Swiss 3T3 cells were separated by SDS PAGE and Western blotted with anti-$G\alpha_{i\text{-}1}$, anti-$G\alpha_{i\text{-}1\text{-}2}$ or anti-$G\alpha_{i\text{-}1\text{-}3}$ antibody, as indicated. (**B**) A recombinant $G\alpha_{i\text{-}1}$ subunit was analysed by SDS PAGE and Western blotted with anti-$G\alpha_{i\text{-}1}$, -$G\alpha_{i\text{-}1\text{-}2}$ or -$G\alpha_{i\text{-}1\text{-}3}$ 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-$G\alpha_{i\text{-}1}$, (**E**, **F**)anti-$G\alpha_{i\text{-}1\text{-}2}$ or(**G**, **H**) anti-$G\alpha_{i\text{-}1\text{-}3}$ antibody. Samples from at least 3 independent experiments were resolved with similar results.
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finding suggested that PMT might inhibit the GTPase activity of $G\alpha_q$, 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 pre-treatment 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 $G\alpha_s$ to inhibit its GTPase activity. Cholera toxin caused an increase in the molecular weight of both the long and short forms of $G\alpha_s$, 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

Table 2. Analysis of pI values of $G\alpha_i$ family isoforms after treatment with PMT.

	Control (pI)			PMT-treated (pI)		
Isoform	**$G\alpha_{i\text{-}1}$**	**$G\alpha_{i\text{-}1,2}$**	**$G\alpha_{i\text{-}1,2,3}$**	**$G\alpha_{i\text{-}1}$**	**$G\alpha_{i\text{-}1,2}$**	**$G\alpha_{i\text{-}1,2,3}$**
i-I	-	5.11±0.01	5.09±0.03	-	5.10±0.01	5.07±0.02
i-II	-	5.22±0.03	5.18±0.03	-	5.17±0.01	5.17±0.02
i-III	-	-	-	-	-	5.34±0.02
i-IV	-	-	5.59±0.03	-	-	5.45±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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GTPase activity in membrane preparations, indicating that PMT inhibits the GTPase activity of $G\alpha_q$ but not G_s (Fig. 6C).

Discussion

PMT executes its cellular effects through the activation of the heterotrimeric G-proteins, G_q, G_{12} and G_i [8–13]. This has been shown to occur in recombinant G_i by PMT-induced deamidation of Gln-205 to glutamic acid, which inhibits its intrinsic GTPase activity [14]. The work we report here 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 $G\alpha_q$ and $G\alpha_{11}$. PMT also stimulated the covalent modification of members of the G_i family. The $G\alpha_{12}$ 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]. $G\alpha_{13}$, but not $G\alpha_{12}$, subunits displayed a reproducible pattern and PMT treatment led to new $G\alpha_{13}$ isoforms at slightly higher pI values. We found no evidence that PMT stimulates the covalent modification of $G\alpha_s$, although the glutamine residue targeted by PMT is conserved in all G-proteins.

Stimulation of G_q-coupled receptors by bombesin only resulted in the detection of the additional $G\alpha_{q/11}$ 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 $G\alpha_{q/11}$ and $G\alpha_i$ 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 G_q in response to PMT [9]. However, pre-treatment of cells with a specific src kinase family inhibitor, SU6656, or a broad spectrum kinase inhibitor, St638, did not prevent PMT from stimulating the covalent modification of $G\alpha_q$ and $G\alpha_i$, 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 $G\alpha$ subunits that is not reversed by the action of phosphatases, which is unlikely.

Deamidation and tyrosine phosphorylation of a $G\alpha$ subunit would have a similar effect on the isoelectric point. The PMT-induced deamidation of in-vitro translated $G\alpha_q$ and recombinant $G\alpha_{i\text{-}2}$ 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 $G\alpha_q$ and $G\alpha_i$ 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 $G\alpha_i$ 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 G_{11}, as PMT could not induce the activation of PLC in G_q-deficient cells [12], and further analysis using $G\alpha_q/G\alpha_{11}$ chimeras also confirmed that PMT did

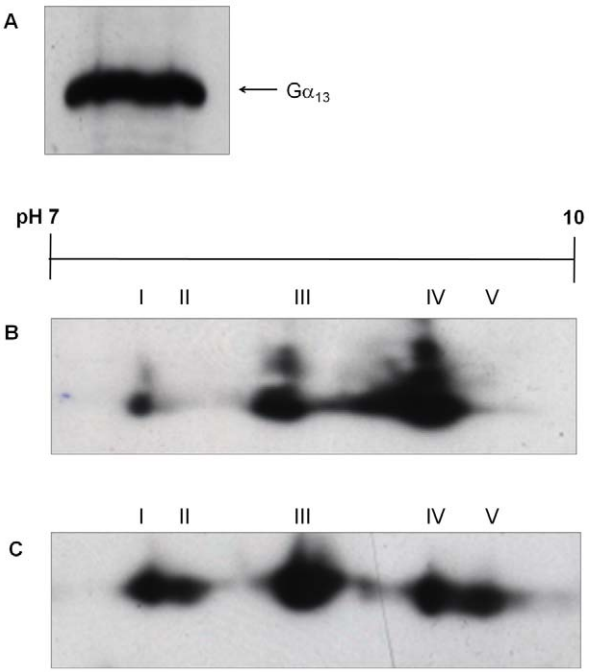

Figure 3. PMT induces the covalent modification of $G\alpha_{13}$. (**A**) Membrane proteins from Swiss 3T3 cells were separated by SDS PAGE and Western blotted with anti-$G\alpha_{13}$ antibody. The location of $G\alpha_{13}$ is indicated. Membrane proteins from Swiss 3T3 cells left (**B**) untreated or (**C**) treated with 150 pM PMT for 4 h were separated by 2-D gel electrophoresis and Western blotted with anti-$G\alpha_{13}$ antibody. Samples from at least 3 independent experiments were resolved with similar results.
doi:10.1371/journal.pone.0047188.g003

Table 3. Analysis of pI values of $G\alpha_{13}$ isoforms after treatment with PMT.

	Control (pI)	PMT-treated (pI)
Isoform	**$G\alpha_{13}$**	**$G\alpha_{13}$**
13-I	8.15±0.02	8.15±0.07
13-II	-	8.24±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).
doi:10.1371/journal.pone.0047188.t003

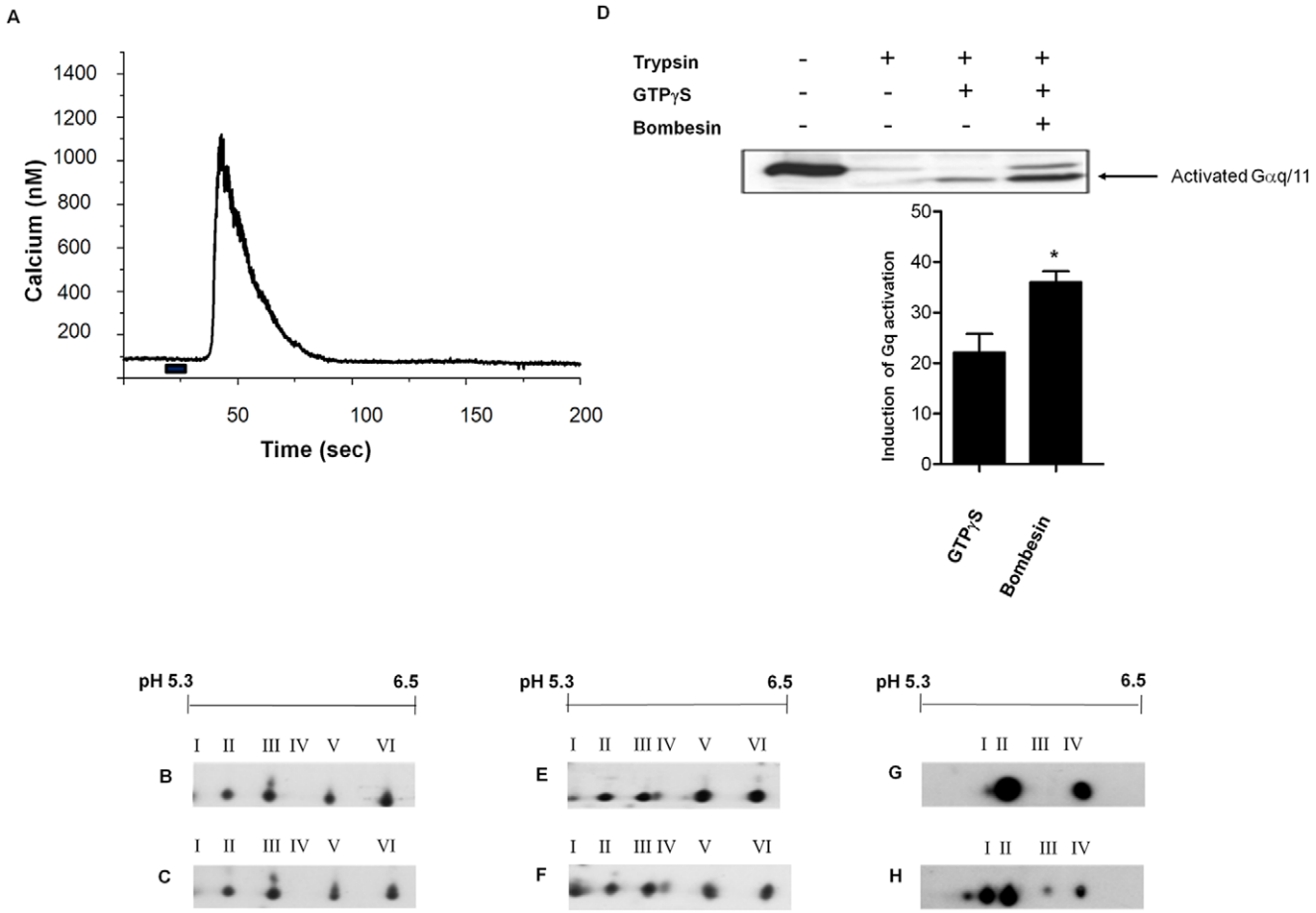

Figure 4. Sodium vanadate treatment mimics PMT effects on $G\alpha_q$ and $G\alpha_i$. (**A**) Indo-1 AM labelled Swiss 3T3 cells were treated with 30 nM bombesin for 10 s (marked with solid bar beneath trace) and intracellular Ca^{2+} release was measured. Membrane proteins from Swiss 3T3 cells that 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-$G\alpha_{q/11}$ 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 $G\alpha_{q/11}$ was separated by SDS PAGE and Western blotted with anti-$G\alpha_{q/11}$ antibody. Quantification of activated $G\alpha_{q/11}$ (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 $G\alpha_{q/11}$ (* $p = 0.002$). 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-$G\alpha_{q/11}$ 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-$G\alpha_{i\text{-}1\text{-}3}$ antibody. Samples from at least 3 independent membrane preparations were resolved with similar results.
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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 $G\alpha_{11}$. $G\alpha_q$ and $G\alpha_{11}$ each contain Gln-209 that is functionally equivalent to Gln-205 in $G\alpha_{i\text{-}2}$ and it would be unlikely that $G\alpha_{11}$ 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 Gα subunits recognised $G\alpha_{11}$ in PMT-treated mouse embryonic fibroblasts that were deficient in $G\alpha_{q/11}$ but transfected to express $G\alpha_{11}$ [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 $G\alpha_{11}$. All the other papers addressing this issue have used the same source of $G_{q/11}$-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 $G\alpha_{13}$ 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-carboxyamide 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

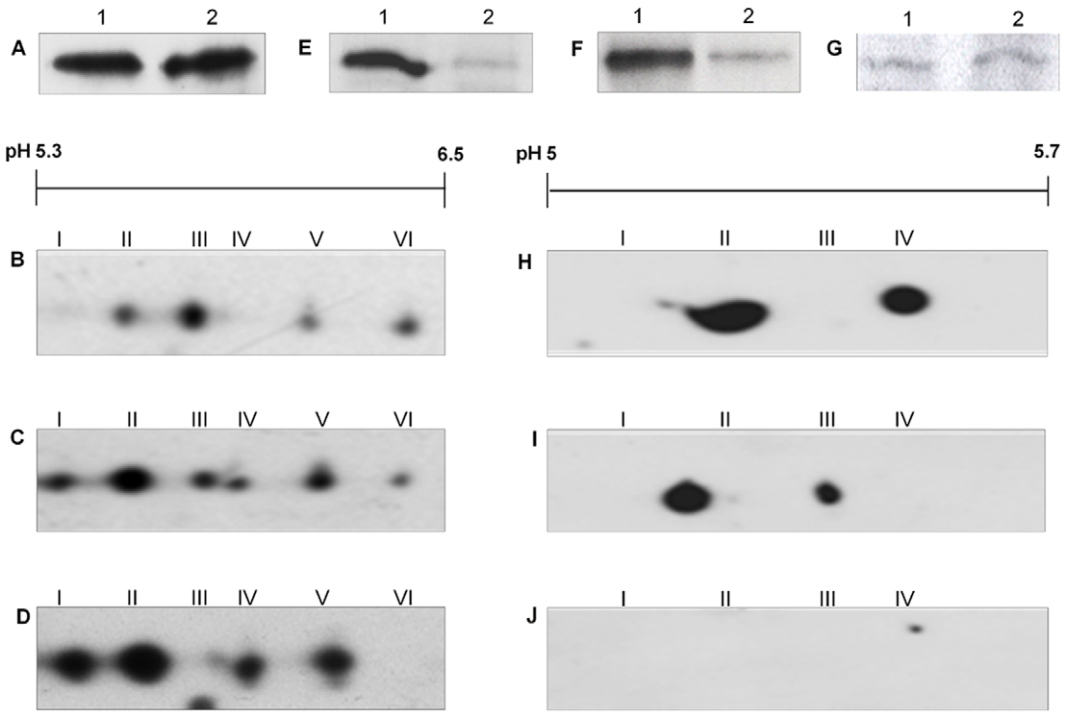

Figure 5. Prolonged exposure of Swiss 3T3 cells to PMT causes the loss of $G\alpha_i$ but not G_q from cell membranes. (**A**) Membrane proteins from Swiss 3T3 cells left untreated (lane 1) or treated with PMT at 1 nM for 16 h (lane 2) were separated by SDS PAGE and Western blotted with anti-$G\alpha_{q/11}$ 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-$G\alpha_{q/11}$ 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-$G\alpha_{i\text{-}1\text{-}3}$ antibody or (**F**) an antibody recognising an internal epitope of $G\alpha_{i\text{-}2}$. (**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-$G\alpha_{i\text{-}1\text{-}3}$ 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-$G\alpha_{i\text{-}1\text{-}3}$ antibody. Samples from 3 independent experiments were resolved with similar results.
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positive charge to produce an alkaline shift, it is possible that PMT preferentially transglutaminates $G\alpha_{13}$ 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 G_s by cholera toxin leads to its downregulation, although ADP-ribosylation of $G\alpha_i$ by pertussis toxin does not result in its degradation [42]. We observed that prolonged treatment of cells with PMT caused the loss of $G\alpha_i$, but not $G\alpha_q$, from membranes prepared from Swiss 3T3 cells. Furthermore $G\alpha_i$ 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 $G\alpha_i$ from its receptor, as the G_i-linked agonist lysophosphatidic acid could not stimulate GTPγS binding to $G\alpha_i$ in membranes derived from these cells [10]. The loss of G_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 $G\alpha_{q/11}$, whereas bombesin could not, which suggested that the action of PMT inhibits the GTPase activity of $G\alpha_{q/11}$. 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 $G\alpha_i$ [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 G_s.

In conclusion, our results demonstrate that treatment of Swiss 3T3 cells with PMT induces the irreversible modification of G-proteins belonging to the G_i and G_q families resulting in an acidic pI shift, which is consistent with the observation that PMT catalyses deamidation of recombinantly expressed G_i causing a similar shift in pI. We found that PMT inhibits the intrinsic GTPase activity of G_q, which complements the finding that PMT-stimulated deamidation of $G\alpha_{i\text{-}2}$ inhibits its GTPase activity. We showed that stimulation of cells with PMT results in the degradation of G_i which provides an explanation for the observation that PMT-treatment blocks G_i activation by a receptor agonist. The unexpected modification of $G\alpha_{11}$ requires further investigation. We demonstrated that PMT treatment causes an alkaline pI shift in $G\alpha_{13}$ and speculate that PMT might preferentially transglutaminate $G\alpha_{13}$. 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.

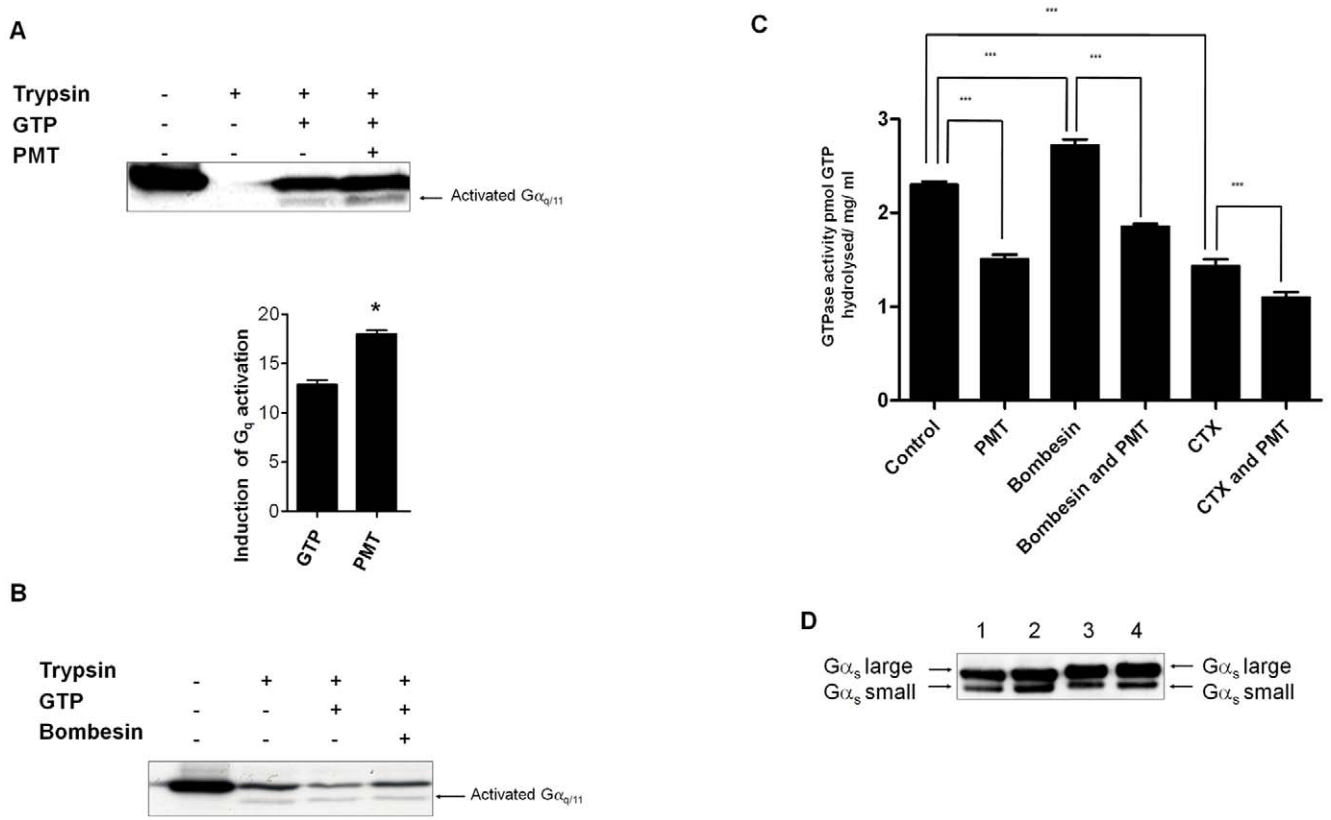

Figure 6. PMT inhibits the GTPase activity of G_q. (**A**) Membrane proteins were incubated in the presence or absence of 150 pM PMT for 1 h with 0.5 nM GTP and tested in a trypsin protection assay as described in Materials and Methods. Proteins were separated by SDS PAGE and Western blotted with anti-$G\alpha_{q/11}$ antibody. Quantification of activated $G\alpha_{q/11}$ (lower panel) was determined by densitometric scanning and the data were analysed using factorial analysis of variance (ANOVA). The induction of activation shown is relative to the density of the band without GTP or PMT. PMT significantly enhanced GTP binding to G_q (* $p = 0.03$). (**B**) Membrane proteins were incubated in the presence or absence of 30 nM bombesin 20 min with 0.5 nM GTP and tested in a trypsin protection assay as described in Materials and Methods. Proteins were separated by SDS PAGE and Western blotted with anti-$G\alpha_{q/11}$ antibody. Samples from at least 3 independent membrane preparations were resolved with similar results. (**C**) Membranes derived from Swiss 3T3 cells that had either been treated or untreated with 150 pM PMT for 4 h or 100 ng cholera toxin, or both, were treated with or without 30 nM bombesin for 20 min in the presence of [γ-^{32}P] GTP. All the experimental conditions were repeated three times, and all data are presented as mean ± standard deviation (SEM). The results for the groups were compared using single-factor analysis of variance (one-way ANOVA), followed by Newnan-Keuls test used to determine differences between groups. Significant changes are indicated by an asterisk (* $P<0.05$, *** $P<0.001$). (**D**) Membranes derived from Swiss 3T3 cells that had either been untreated (lane 1) or treated with 150 pM PMT for 4 h (lane 2), or 100 ng cholera toxin for 16 h (lane 3), or both PMT and cholera toxin (lane 4) were resolved by SDS PAGE followed by Western blotting with an anti-$G\alpha_s$ antibody. Samples from at least 3 independent membrane preparations were resolved with similar results.
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Supporting Information

Figure S1 PMT does not induce the covalent modification of $G\alpha_s$. (**A**) Membrane proteins from Swiss 3T3 cells were separated by SDS PAGE and Western blotted with anti-$G\alpha_s$ 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-$G\alpha_s$ antibody. Samples from at least 3 independent experiments were resolved with similar results.
(TIF)

Figure S2 Kinase inhibitors do not block PMT induced modification of $G\alpha_{q/11}$ or $G\alpha_i$. 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 blotting 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 (**C–F**) anti-$G\alpha_{q/11}$ antibody or (**G–J**) anti-$G\alpha_{i\text{-}1\text{-}3}$ antibody. Samples were resolved from 2 independent experiments with similar results.
(TIF)

Figure S3 Mutant PMT does not induces the covalent modifications of $G\alpha_q$ or $G\alpha_i$. Membrane proteins from Swiss 3T3 cells (**A, C**) left untreated or (**B, D**) treated with 150 pM PMT^{C1165S} for 4 h, separated by 2-D gel electrophoresis and Western blotted with either (**A, B**) anti-$G\alpha_{q/11}$ or (**C, D**) anti-$G\alpha_{i\text{-}1\text{-}3}$ antibodies. Samples from 3 independent experiments were resolved with similar results.
(TIF)

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

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

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Author Contributions

Conceived and designed the experiments: RB KH JR AL. Performed the experiments: RB KH JR. Analyzed the data: RB KH JR AL. Contributed reagents/materials/analysis tools: RB KH JR. Wrote the paper: RB KH JR AL.

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