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Pasteurella multocida toxin as a tool for studying G_q signal transduction

B. A. Wilson and **M. Ho**
Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA

Abstract

Pasteurella multocida toxin (PMT) stimulates and subsequently uncouples phospholipase C (PLC) signal transduction through its selective action on the $G\alpha_q$ subunit. This review summarizes what is currently known about the molecular action of PMT on G_q and the resulting cellular effects. Examples are presented illustrating the use of PMT as a powerful tool for dissecting the molecular mechanisms involving pertussis toxin (PT)-insensitive heterotrimeric G proteins.

Introduction

Protein toxins have long been known to constitute important virulence determinants for pathogenic bacteria. The anthrax bioterrorism events of 2001 and the emergence of antibiotic-resistant, toxin-producing bacteria have provided strong impetus to increase our understanding of toxin-mediated disease processes (Wilson and Salyers 2002). The growing relevance of toxin-mediated effects on host cells in developing alternative antitoxin strategies heightens the need to better understand the structure-function relationships of protein toxins produced by pathogenic bacteria. Over the past couple of decades, our understanding of toxin action has expanded tremendously. Information learned from these efforts has enabled scientists to exploit their noxious properties for beneficial applications in studying problems in cell biology, physiology, and pharmacology.

Many protein toxins share the common feature of being highly specialized enzymes, capable of entering eukaryotic cells and catalyzing reactions that interfere with normal signal transduction and physiological processes, often resulting in morphological changes, cellular damage or cell death. Because of their highly specific action in cells, bacterial protein toxins can be used as selective and efficient tools for studying molecular mechanisms controlling signal transduction and other physiological processes (Schiavo and Van Der Goot 2001). For example, a number of toxins, including the clostridial toxins C2, C3, toxA and toxB, and the *E. coli* toxins CNF1 and CNF2, among others, have been used as selective modulators of cytoskeletal function through their action on small GTPases (Aktories et al. 2000). The clostridial neurotoxins have been enormously beneficial in uncovering molecular mechanisms of neurotransmitter release and essential aspects of neuronal physiology (Lalli et al. 2003; Schiavo et al. 2000). These neurotoxins are increasingly being put to beneficial use in medicine for the treatment of human diseases characterized by hyperfunction of nerves (Rossetto et al. 2001).

Heterotrimeric G proteins constitute a large family of pivotal regulatory GTPases that are responsible for transducing external (e.g., hormonal) signals from ligand-bound receptors to intracellular responses. They are made of α, β, and γ subunits and are distinguished into four main classes: G_s, G_i, G_{12}, and G_q. The first toxins used to define the molecular mechanisms

e-mail: bawilson@life.uiuc.edu, Tel.: +1-217-2449631, Fax: +1-217-2446697.

of heterotrimeric G proteins and adenylate cyclase-mediated signaling pathways were cholera toxin (CT) and pertussis toxin (PT): CT activates G_s proteins, while PT inhibits $G_{i/o}$ proteins (Casey and Gilman 1988). PT was used to further distinguish a group of PT-insensitive G proteins (see Table 1) (Fields and Casey 1997). Of these G proteins that are refractory to PT treatment, a subgroup, later identified as the G_q family, were found to play critical roles as regulators of phospholipase C (PLC) signaling (Fields and Casey 1997; Rhee and Choi 1992; Sternweis and Smrcka 1992).

Receptors for many hormones, neurotransmitters and growth factors are coupled to G_q proteins. Stimulation of G_q-coupled receptors results in transient elevation of intracellular Ca^{2+} and increased levels of inositol trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers control vital cellular processes, including fertilization, cell growth, transformation, secretion, muscle contraction, metabolism and sensory perception (Berridge 1993). IP_3 and DAG are generated from phosphatidylinositol 4,5-bisphosphate (PIP_2) through the action of PLC, of which γ-isoforms are activated through tyrosine kinase-linked receptors and β-isoforms are activated through G-protein-coupled receptors (Rhee and Choi 1992; Sternweis and Smrcka 1992). Ligands and G-protein-coupled receptors that activate PLCβ (Deckmyn et al. 1993; Quick et al. 1994; Rebecchi and Pentyala 2000; Rhee and Choi 1992; Sternweis and Smrcka 1992) can be distinguished by their sensitivity to PT (Berstein et al. 1992a, b; Camps et al. 1992; Wu et al. 1993). The βγ subunits (but not α subunits) of PT-sensitive $G_{i/o}$ proteins preferentially stimulate PLCβ2>PLCβ3>PLCβ1, whereas the α subunits of the PT-insensitive G_q proteins stimulate PLCβ1≧PLCβ3≫PLCβ2 (Hepler et al. 1993; Park et al. 1993; Rhee and Choi 1992; Smrcka and Sternweis 1993).

Until recently, there had been no specific modulating reagent available for studying the role of G_q proteins in hormonal communication and signal transduction. The dermonecrotic toxin produced by *Pasteurella multocida* (PMT) can now be added to the list of bacterial protein toxins that modulate G proteins (Table 1). PMT stimulates Ca^{2+} and IP_3 signaling by activating G_q-dependent PLCβ1 (Wilson et al. 1997). PMT facilitation of $G\alpha_q$-protein coupling to PLCβ1 causes the same cellular responses elicited by G_q-protein-linked receptors, such as the muscarinic (M_1, M_3, M_5), bombesin, vasopressin, endothelin, thyrotropin-releasing hormone (TRH), and adrenergic receptors (α_1AR). Understanding the bio-chemical mechanism of PMT action may thus afford unique insight into G_q-mediated molecular signaling events and enables the use of PMT as a tool for studying these processes.

Cellular effects of PMT

PMT is the major virulence factor produced by *P. multocida* that is responsible for atrophic rhinitis, pneumonia-like respiratory disease, and dermonecrosis (Foged 1992). PMT is secreted as a monomeric, 1285-amino acid protein (M_r 146-kDa) (Lax and Chanter 1990; Petersen 1990; Petersen and Foged 1989). PMT binds to ganglioside-type receptors and enters mammalian cells via receptor-mediated endocytosis (Dudet et al. 1996; Pettit et al. 1993; Rozengurt et al. 1990) and acts intracellularly to initiate a number of signaling pathways leading to DNA synthesis and cellular proliferation (Higgins et al. 1992; Rozengurt et al. 1990; Seo et al. 2000; Wilson et al. 1997, 2000). Purified PMT alone is sufficient to experimentally induce progressive atrophic rhinitis in swine and symptoms of pneumonia in rabbits (Chrisp and Foged 1991; Foged 1992; Lax and Chanter 1990). Recombinant PMT is indistinguishable from native toxin, and both are equally potent at pi-comolar concentrations (Lax and Chanter 1990; Rozengurt et al. 1990; Wilson et al. 1997). Vaccination against PMT protects against challenge with *P. multocida* and development of atrophic rhinitis (Foged 1992).

PMT stimulates osteoclastic bone resorption in vitro (Felix et al. 1992; Kimman et al. 1987) and increases osteoclast cell number in vivo (Martineau-Doize et al. 1993). PMT appears to

stimulate the differentiation of preosteoclasts into osteoclasts (Jutras and Martineau-Doize 1996) and promotes osteoclast proliferation leading to bone resorption, while apparently inhibiting bone regeneration by osteoblasts (Mullan and Lax 1998; Sterner-Kock et al. 1995). PMT acting on these multiple cell types, including activating mature osteoclasts and inducing preosteoclast proliferation, as well as proliferation/differentiation of periosteal (fibroblastic, osteogenic, and adipogenic) cells, may contribute to the symptoms of atrophic rhinitis (Mullan and Lax 1998; Rozengurt et al. 1990). Recent evidence suggests that immunomodulation of the host may be an additional function of the toxin important in pathogenesis (Jordan et al. 2003).

In cultured epithelial cells, such as calf or monkey (Vero) kidney cells, calf testis, or bovine embryonic lung (EBL) cells, PMT causes primarily morphological changes and cytotoxic effects (Pennings and Storm 1984; Pettit et al. 1993; Rutter and Luther 1984). In cultured mesenchymal cells, such as murine, rat, or human fibroblasts, and in osteoblasts, PMT action is mitogenic and initiates DNA synthesis and cell division (Mullan and Lax 1998; Rozengurt et al. 1990). PMT has also been shown to induce anchorage-independent cell growth of fibroblasts (Higgins et al. 1992), as evidenced by colony formation in soft agar, suggesting that it has the ability to promote a transformed phenotype and leading to the speculation that it could promote tumor formation and cancer (Lax and Thomas 2002). Flow cytometry analysis of cells treated with PMT showed that PMT stimulates cells to move from the G_1 phase into and through the S phase, but it does not trigger apoptosis (Wilson et al. 2000). PMT-treated confluent quiescent Swiss 3T3 cells formed dense monolayers over the course of 4–6 days, with a concomitant increase in cell number up to threefold (Wilson et al. 2000). However, cell cycle analysis revealed that after the initial mitogenic response to PMT, cells subsequently arrested primarily in G_1 and became unresponsive to further PMT treatment (see Fig. 1), indicating that the mitogenic response was not sustained (Wilson et al. 2000).

Western blot analysis of the effect of PMT on the expression of a number of cell cycle markers, including the proto-oncogene c-Myc; cyclins D1, D2, D3, and E; p21; PCNA; and the Rb proteins, p107 and p130, showed that PMT initially upregulated these markers and stimulated cell cycle progression in Swiss 3T3 cells, yet continued expression of these markers, and hence continued proliferation, was not sustained (Wilson et al. 2000). However, PMT exhibited a differential effect on epithelial-like cells. Confluent Vero cells underwent rapid, dramatic morphological changes upon toxin exposure, but a mitogenic effect was not evident, based on the lack of a PMT-induced increase in cell numbers or in the rate of DNA synthesis, which was further substantiated by flow cytometry analysis. Furthermore, PMT failed to upregulate PCNA or cyclins D3 and E, which is critical for driving cells from G_1 into S phase, and hence, little or no cell cycle progression occurred in Vero cells (Wilson et al. 2000).

PMT effects on signal transduction

PMT and G_q-PLC signaling

PMT activates inositol phosphate pathways, Ca^{2+} mobilization and PKC-dependent phosphorylation in cultured fibroblasts and osteoblasts (Mullan and Lax 1998; Staddon et al. 1990, 1991). PMT also potentiates G protein-coupled receptor responses to bombesin, vasopressin, and endothelin (Murphy and Rozengurt 1992). These effects suggested the involvement of a cellular phosphatidylinositol-specific phospholipase C (PLC) in PMT action (Murphy and Rozengurt 1992). Wilson et al. (Wilson et al. 1997) subsequently demonstrated direct PMT-mediated stimulation of PLCβ1 activity and IP_3-induced intracellular Ca^{2+} mobilization by using voltage-clamped *Xenopus* oocytes as a model system to monitor the transient Ca^{2+}-dependent Cl^- current evoked upon microinjection with PMT. To identify the intracellular targets involved in the PMT-induced IP_3 signaling pathway, they examined the effects of specific antibodies against various G-protein and PLC signaling molecules on the

PMT-induced Cl^- currents in the oocyte system. As showed in Fig. 2, only antibodies directed against PLCβ1, $G\alpha_{pan}$, and the C-terminus of $G\alpha_{q/11}$ completely blocked the PMT-mediated response in oocytes. In addition, GDPβS, a known inhibitor of Gα subunit-mediated signaling pathways, blocked the PMT-induced response. Further-more, overexpression of mouse $G\alpha_q$ in *Xenopus* oocytes increased the PMT-induced response, whereas treatment with antisense $G\alpha_q$ mRNA reduced the response. These results established the direct involvement of $G\alpha_q$ protein in PMT-activation of PLCβ1 (Fig. 2).

Antibodies against Gβ subunit did not block, but rather enhanced the PMT-induced response (Fig. 2), distinguishing that PMT action did not involve Gβγ subunit activation of PLCβ2 by a $G_{i/o}$-dependent pathway. Rather, by binding to Gβ, the antibodies caused the dissociation of $G\alpha_q$ subunit from the heterotrimeric complex, which could then be acted upon by PMT to give an enhanced response. The PMT-induced response was likewise enhanced by the release of $G\alpha_q$ subunit through sequestration of Gβγ subunits by using PT (Wilson et al. 1997). From these studies, the researchers concluded that the monomeric $G\alpha_q$ subunit is the preferred target of PMT action, which subsequently activates PLCβ1. Mouse knockout cell lines were used to confirm that PMT-induced formation of inositol phosphates was exclusively dependent on $G\alpha_q$, and not closely-related G_q family members, such as G_{11}, G_{12}, or G_{13} (other G_q-family members, G_{14} or $G_{15/16}$, were not examined) (Zywietz et al. 2001).

A recent study using a series of chimeras between $G\alpha_q$ and $G\alpha_{11}$ in $G\alpha_{q/11}$-knockout cells identified a region of the helical domain of $G\alpha_q$ that is important for PMT-induced activation of PLCβ (Orth et al. 2004). Exchange of Glu-105 or Asn-109 of $G\alpha_{11}$, each of which is located in the helical domain of the Gα subunit, with the corresponding His residues of $G\alpha_q$ resulted in a mutant $G\alpha_{11}$ that was now capable of mediating PMT-induced activation of PLCβ. However, the converse was not true, in that the reciprocal exchange of either His in $G\alpha_q$ with the corresponding $G\alpha_{11}$ amino acid did not prevent PMT activation of PLCβ. Whether this differential interaction is due to a difference in PMT recognition of $G\alpha_q$ versus $G\alpha_{11}$, due to a difference in $G\alpha_q$ versus $G\alpha_{11}$ recognition of PLCβ, or due to a difference in recognition of $G\alpha_q$ versus $G\alpha_{11}$ by another unidentified PMT mediator has yet to be determined.

PMT was shown to stimulate tyrosine phosphorylation of $G\alpha_q$, but a mutant of PMT that does not activate G_q was also found to cause tyrosine phosphorylation of G_q, suggesting that this phosphorylation is not a prerequisite for $G\alpha_q$ activation by PMT (Baldwin et al. 2003). Consequently, although tyrosine phosphorylation of $G_{q/11}$ has been reported to regulate $G_{q/11}$ activation (Umemori et al. 1997, 1999), the role of tyrosine phosphorylation in PMT action on G_q is not clear.

Repeated microinjection of IP_3 into oocytes reproduced transient Ca^{2+}-dependent Cl^- currents, indicating that the IP_3 pathway is not readily desensitized. After the initial transient response to PMT, additional injection of IP_3 still gave a response, but additional injection of PMT had no further response (Wilson et al. 1997). These results confirmed that the target of PMT action is upstream of IP_3 release and that PMT uncouples the signaling between G_q and PLCβ1. A model for the intracellular action of PMT based on all of these results is shown in Figure 3.

PMT and downstream signaling

Some of the intracellular events that occur upon exposure to PMT are: enhanced hydrolysis of inositol phospholipids to increase the total intracellular content of inositol phosphates (Staddon et al. 1991); increased production of DAG (Staddon et al. 1990); mobilization of intracellular Ca^{2+} pools (Staddon et al. 1991); interconversion of GRP78/BiP (Staddon et al. 1992); and activation of protein kinase phosphorylation (Lacerda et al. 1996; Staddon et al. 1990). It has been suggested that activation of the small Rho GTPase mediates PMT-induced tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$) and paxillin, which results in actin stress

fiber formation and focal adhesion assembly (Lacerda et al. 1996; Ohnishi et al. 1998; Thomas et al. 2001). Yet, this tyrosine phosphorylation appears to be independent of PKC activation and Ca^{2+} mobilization (Lacerda et al. 1996; Ohnishi et al. 1998).

How PMT-mediated activation of the PLC-IP_3 signaling pathway promotes cytoskeletal rearrangement is, as of yet, not clear. One hint toward this may be the recent finding that PMT can associate with vimentin (Shime et al. 2002), a component of intermediate filaments in cells. Another possibility is for PMT to act on the actin cytoskeleton through its indirect action on Rho via G_q (Chikumi et al. 2002; Dutt et al. 2002; Katoh et al. 1998; Vogt et al. 2003). Although the stimulation of inositol phosphate signaling by PMT did not occur in $G\alpha_q$-deficient or $G\alpha_q/G\alpha_{11}$-deficient cells, PMT could still stimulate other cellular effects in those knockout cells, including Rho activation, Rho-dependent actin rearrangements and focal adhesions, as well as JNK and Erk mitogenic signaling (Zywietz et al. 2001). These results indicate that certain effects of PMT action may also occur through other signaling pathways, independent of G_q or G_{11}. One possibility is that PMT acts on G_{12} or G_{13}, both of which are known to activate Rho protein (Fukuhara et al. 1999; Gratacap et al. 2001; Hart et al. 1998; Kozasa et al. 1998; Kurose 2003). $G\alpha_{12/13}$ can induce Rho-dependent responses by interaction with Rho-specific guanine nucleotide exchange factors (Sah et al. 2000). Although it is not known if PMT acts on $G\alpha_{12/13}$, this may provide a mechanism by which PMT could activate Rho in the absence of G_q and G_{11}.

PMT as a tool for studying signal transduction

PMT as a tool for studying G_q-PLC signaling

A number of investigators have used PMT as a pharmacological tool to study G_q-coupled PLC signaling along the lines of that shown in Figure 3. For example, PMT has been used as a selective activator of G_q-coupled PLC effectors. G_q-coupled adrenergic receptor signaling in cardiomyocytes differs significantly between even closely related animal species, such as mice and rats. In the rat cardiomyocytes, α_1-AR and endothelin receptors selectively activated PLC through G_q protein, but these receptors were not functional in mouse cardiomyocytes. PMT was used to show that G_q-PLC signaling pathway was still functional in the mouse cardiomyocytes (Sabri et al. 2000).

In discriminating PT-insensitive G-protein coupling of noradrenaline-induced α_{1A}AR activation in neonatal rat cardiomyocytes, α_{1A}AR was found to couple specifically to $G_{q/11}$ and not $G_{12/13}$ proteins by showing that overexpression of $G_{q/11}$-specific RGS4, but not $G_{12/13}$-specific Lsc-RGS blocked α_{1A}AR activation of both PLC and phospholipase D (PLD) (Gosau et al. 2002). In addition, this study showed that PLD activation occurred subsequent to G_q-activation of PLCβ and novel, Ca^{2+}-independent PKC isoforms δ and ε. The importance of G_q and not G_{11} in α_{1A}AR activation of both PLC and PLD was further demonstrated by using PMT, which mimicked the α_{1A}AR response.

Histamine induces catecholamine secretion from bovine adrenal chromaffin cells. PMT treatment caused a substantial additive increase in basal and histamine-stimulated inositol phosphate levels, but did not increase or prevent basal or histamine-stimulated secretion of the catecholamines, adrenaline and noradrenaline (Donald et al. 2002). This study showed that the secretion occurs through a PLC-independent membrane depolarization. The results obtained with PMT were consistent with other data, which showed that the PLC inhibitor ET-18-OCH3 blocked inositol phosphate formation without inhibiting catecholamine secretion. This histamine-induced catecholamine secretion also does not involve Ca^{2+} mobilization, since IP_3-receptor inhibitors, such as 2-aminoethoxydiphenylborate (2-APB) or ryanodine plus caffeine, or thapsigargin-depletion of intracellular Ca^{2+} stores, had no effect.

PMT was used to discriminate between G_q-dependent and G_q-independent signaling induced by saccharin in isolated rod taste cells from frogs (Okada et al. 2001). Data had suggested a role for G-protein-mediated release of IP_3 rather than cAMP in the saccharin- induced cationic conductance. However, treatment with PMT did not induce a response in the frog taste cells, suggesting that it may be a Gβγ-coupled PLCβ2 isoform rather than a $G\alpha_q$-coupled PLCβ1 or PLCβ3 isoform that is involved in saccharin taste transduction. Although this conclusion has since been substantiated by others (Imendra et al. 2002), a cautionary note might be warranted here, regarding interpreting negative results with the use of PMT as a reagent. The investigators had found that PMT did not elicit any current response in the cells (Okada et al. 2001); however, unlike the previous studies mentioned above, they had not verified as a control that PMT in their system was still able to evoke an IP_3 response.

PMT has been used to explore the pathways that mediate interaction between endogenous $G\alpha_q$ and Rho signaling. In COS-7 cells, $G\alpha_q$ coimmunoprecipitated with the Rho guanine nucleotide exchange factor (Lbc), and $G\alpha_q$ and Lbc synergistically activated serum response element (SRE.L)-dependent gene expression in a PLC- and Rho-dependent manner (Sagi et al. 2001). In this study, the ability of PMT to synergize with Lbc in stimulating SRE.L-mediated gene expression confirmed that $G\alpha_q$ at endogenous levels also interacts with Rho-GEF-regulated pathways. However, it is still not clear how $G\alpha_q$ activation leads to Rho activation since, unlike for $G_{12/13}$, expression of $G\alpha_q$ alone did not activate Rho, but did enhance Rho-dependent responses.

PMT has also been used to help define the signaling processes that control maturation of dendritic cells (DCs) (Bagley et al. 2004). In these studies, the investigators were interested in determining the role of PLC and Ca^{2+} signaling in activation of monocyte-derived DCs. To show the involvement of Ca^{2+} signaling in DC maturation, they used a number of different agonists, including lipopolysaccharide, CT, dibutyryl-cAMP, prostaglandin E2, and the Ca^{2+} ionophore A23187, all of which induced maturation. PMT was employed as a control to validate the involvement of PLC signaling in DC maturation. They further showed that this activation by PMT was inhibited by xestospongin, an inhibitor that blocks Ca^{2+} release from IP_3-gated intracellular stores.

PMT as a tool for studying circadian rhythms

PMT has recently been used as a tool to study cholinergic regulation of the circadian clock in the hypothalamic suprachiasmatic nucleus (SCN) of the rat brain. In a brain slice model, the SCN clock is subject to muscarinic regulation through the G_q-coupled M_1 mAChR, with sensitivity exhibited only during the night phase of the clock's 24-h cycle (Gillette et al. 2001). It was found that the effect of 1-h treatment with PMT on circadian clock resetting mimicked the advance of the clock phase induced by carbachol-stimulated M_1 mAChR signaling and IP_3-mediated Ca^{2+} release (L. Artinian, W. Yu, B.A. Wilson, E. Gratton, M.U. Gillette, unpublished data). Inhibitors of IP_3-mediated Ca^{2+} release, such as xestospongin, blocked this PMT-induced phase shift. Thus, PMT-mediated activation of the G_q-PLC-IP_3-induced Ca^{2+} release resets the clock in the same direction as activation of muscarinic receptors and cGMP signaling in early night.

PMT as a tool for studying endothelial permeability

PMT has been used to show that Rho activation and resulting cell retraction plays an important role in increased endothelial permeability (Essler et al. 1998). Disruption of endothelial integrity by PMT involves Rho-dependent activation of Rho kinase (ROKα), which in turn inactivates myosin light chain (MLC) phosphatase PP1 and thereby increases MLC phosphorylation and actin reorganization, followed by cell retraction and concomitant rise in endothelial permeability (Fig. 4). PMT-induced actin rearrangement could be blocked by

microinjection of the Rho GTPase inhibitor C3 transferase from *C. botulinum* or microinjection of the Rho-binding domain (RBD) or pleckstrin homology (PH) domain of ROKα, which interfere with ROKα interaction with its regulators. These results have led to the speculation that PMT-mediated Rho activation is responsible for the observed vascular effects of PMT in bite wounds (Aepfelbacher and Essler 2001).

PMT as a tool for studying GIRK signaling

After the initial stimulatory response, PMT effectively uncouples G_q-PLC signaling and prevents any further activation through G_q (Wilson et al. 1997, 2000). Several investigators have demonstrated the effectiveness of using prolonged treatment with PMT to down-regulate G_q-mediated signaling. In heart tissue and in various neuronal and endocrine cells, cellular excitability is regulated by G-protein-coupled inward rectifying K^+ (GIRK) channels through selective hormonal stimulation (Sadja et al. 2003). As illustrated in Figure 5, GIRKs are activated in a PT-sensitive manner by receptors coupled to G-proteins of the $G_{o/i}$ family, such as the G_i-coupled A_1 adenosine receptor. This activation results from binding of the G_i-protein βγ subunits to the channel. On the other hand, GIRK currents can be inhibited in a PT-insensitive manner either by $G_{i/o}$-coupled receptors, such as M_2/M_4 mAChR, or by G_q-coupled receptors, such as α_{1A}AR or M_1/M_3 mAChR. However, it was not clear whether the inhibition of GIRKs by these two different receptor types occurred through the same mechanism (Fig. 5).

To address this question, PMT was used to demonstrate the existence of two different regulatory pathways for PT-insensitive inhibition of GIRK channels, one involving PMT-insensitive $G_{i/o}$ and the other involving PMT-sensitive G_q (Fig. 5) (Buenemann et al. 2000). The researchers examined the effect of prolonged PMT pretreatment on GIRK inhibition in human embryonic kidney HEK293 cells transfected with GIRK1/4, A_1 adenosine receptors, and G_q-dependent or $G_{i/o}$-dependent inhibitory receptors. They found that pretreatment with PMT did not prevent the Gβγ-mediated GIRK activation by stimulatory A_1 adenosine receptors. Likewise, PMT treatment did not affect the $G_{i/o}$-coupled M_2 mAChR-mediated inhibition of GIRK. In contrast, PMT completely blocked the inhibition of GIRK by the $G\alpha_q$-coupled α_{1A}AR receptor.

PMT has also been used to mediate uncoupling of G_q signaling in cardiomyocytes to specifically block GIRK inhibition induced by phenylephrine and endothelin-1 (Meyer et al. 2001). In these studies, the investigators provided strong evidence for use of PMT as a superior tool to the commonly used aminosteroid PLC inhibitor U73122 for demonstrating the involvement of G_q-coupled PLCβ activity in mediating GIRK inhibition through PLCβ-mediated depletion of PIP_2. These conclusions were further substantiated by results in which PMT was used to examine GIRK channel regulation in HEK293 cells coexpressing GIRK1/4 with the $G_{i/o}$-coupled 5-HT_{1A} serotonin or G_q-coupled thyrotropin-releasing hormone (TRH) receptors (Lei et al. 2001). Both TRH and constitutively active $G\alpha_q$ inhibited GIRK. On the other hand, the inhibition of GIRK by TRH was shutdown by prolonged pretreatment with PMT, as well as by treatment with other known inhibitors of G_q signaling, RGS2 and PLCβ1-ct, which bind to $G\alpha_q$ and interfere with $G\alpha_q$-effector interaction, or by treatment with agents known to lower plasma membrane PIP_2 levels via cleavage of PIP_2 with 5′-phosphatidylinositol-phosphatase or via sequestration of PIP_2 with PLCδ-PH.

PMT as a tool for studying mitogenic signaling

Because PMT acts on G_q, PMT can now be used to study the role of G_q-mediated signaling in hormonal-stimulated mitogenesis. PMT stimulation of Erk signaling was shown to occur via G_q-dependent transactivation of the epidermal growth factor (EGF) receptor in a Ras-dependent manner in some cells, but via a PKC-dependent, Ras-independent pathway in other

cells (Fig. 6). In one study (Seo et al. 2000), the mechanism of PMT-mediated Erk activation was compared to that of endogenous $G_{q/11}$-protein-coupled α-thrombin receptors in HEK-293 cells. Both PMT and the endogenous G_q-coupled receptors were found to induce Ras-dependent Erk activation via a PKC-independent transactivation of the EGF receptor. For both PMT and the α-thrombin receptor, expression of two inhibitors of G_q signaling, a dominant-negative mutant of the G-protein-coupled receptor kinase (GRK2) and a C-terminal peptide of $G\alpha_q$ ($G\alpha_{q305–359}$), blocked Erk activation. Erk activation by PMT was insensitive to a PKC inhibitor (GF109203X), but was blocked by an EGF receptor-specific inhibitor tyrphostin (AG1478), as well as by dominant-negative inhibitors of mSos1 and Ha-Ras. The results suggested that PMT-activated $G\alpha_q$ transactivates the EGF receptor. In the other study involving cardiac fibroblasts, PMT was also found to stimulate Erk activation via EGF receptor transactivation (Sabri et al. 2002). However, in cardiomyocytes novel PKC isoforms mediated PMT activation of Erk1/2, as well as p38-MAPK, and JNK, and the EGF receptor appeared to have no role in this activation (Fig. 6).

Another example of PMT-mediated transactivation of tyrosine kinase signaling via G_q activation was reported for the generation of inositol phosphoglycans, second messengers of insulin signaling (Sleight et al. 2002). In rat liver membranes, PMT stimulated the production of inositol phosphoglycans, as measured by release of myoinositol and chiroinositol after acid hydrolysis, in a manner similar to what occurs upon insulin stimulation. Interaction between the G_q signaling pathway and the insulin receptor tyrosine kinase pathway was further supported by immunogold-labeling experiments showing colocalization of the insulin receptor β subunit (IRβ) and $G\alpha_{q/11}$ in partially purified rat liver membranes, enriched in PLCβ1, clathrin, and caveolin-1 (Sleight et al. 2002). Furthermore, direct interaction of $G\alpha_{q/11}$ with IRβ was demonstrated in another study through coimmuno-precipitation (Imamura et al. 1999).

PMT as a tool for studying apoptosis

In neonatal rat cardiomyocytes, PMT induced cardiac hypertrophy (i.e., cardiomyocyte enlargement, sarcomeric organization, and atrial natriuretic factor expression) in a manner similar to that which occurs upon norepinephrine stimulation (Sabri et al. 2002). PMT also activated Erk, and to a lesser extent p38 MAPK and JNK, via activation of PLC and novel PKC isoforms. PMT decreased basal Akt activation by preventing Akt phosphorylation through the activation by EGF or insulin-like growth factor-1 (IGF-1), and consequently enhanced cardiomyocyte susceptibility to apoptotic agents such as H_2O_2. PMT initially stimulates cardiac hypertrophy in a manner similar to moderate G_q stimulation, yet inhibits the Akt survival pathway and thereby enhances cardiomyocyte susceptibility to apoptosis in a manner similar to what occurs under intense, prolonged stimulation of G_q-coupled receptors (Adams et al. 1998). This suggests that hypertrophy and apoptosis may represent two phases of the same process leading eventually to cardiac decompensation and heart failure and further suggests that PMT might serve as an excellent tool to study this process.

A connection between G_q-coupled α_{1A}AR receptor activation and augmented UV-induced apoptosis through inhibition of phosphatidylinositol 3-kinase (PI3K) and Akt in response to platelet-derived growth factor (PDGF), as well as insulin and insulin-like growth factor 1 (IGF-1), has also come to light (Ballou et al. 2000, 2001). In Rat-1 fibroblasts, PI3K signaling induced by PDGF was also inhibited by treatment with PMT in a manner similar to α_{1A}AR (Fig. 6) (Lin et al. 2003). In this study, PMT pretreatment reduced the amount of phospho-Tyr751 on the PDGF receptor β subunit, thereby eliminating the docking site for the p85 subunit of PI3K. In addition, PMT pretreatment significantly inhibited the PDGF-induced Akt phosphorylation at Ser473.

Conclusions and future prospects

Discriminating among the various G-proteins involved in signal transduction processes has always been a challenge for cell biologists, physiologists, and pharmacologists. The discovery that certain toxins produced by bacteria can selectively act on different G-proteins has provided researchers with a growing repertoire of agents that can be used to manipulate signaling pathways for elucidating cellular functions of various signaling molecules. Until recently, studying the signaling of G_q GTPases was relatively intractable due to the lack of effective molecular tools that were specific for them; in fact, this family was referred to simply as the PT-insensitive G-proteins. While we do not yet know the precise biochemical basis for PMT action on G_q, there is considerable and convincing evidence that PMT can be used as a highly selective agent that targets G_q protein-coupled PLC signal transduction. Because initial exposure to PMT results in activation of G_q signaling, but prolonged treatment subsequently uncouples G_q-dependent PLC signaling, PMT can be used as both an activator and a downregulator of G_q-PLC signaling, depending upon the length of toxin treatment. PMT has already been shown to decipher a number of important signaling pathways involving G_q signaling. With the introduction of PMT as a G_q-selective molecular tool for studying PLC-mediated signaling, we can now begin to discern the different roles that G_q family members play in other signal transduction pathways and physiological processes. An important question that still remains to be answered is whether the other G_q family members, which are not coupled to PLC, might also be targets of PMT. Indeed, there is strong evidence for other PMT effects that are independent of G_q-PLC signaling in G_q- and $G_{q/11}$-knockout cells.

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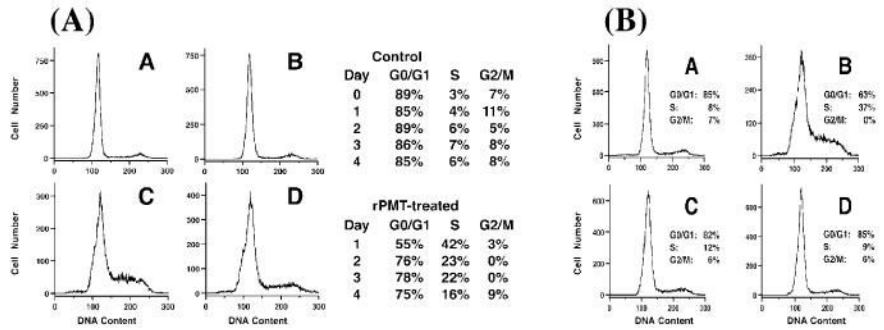

Fig. 1.

Effect of PMT treatment on cell cycle progression in Swiss 3T3 cells. Shown are results from flow cytometry analyses of confluent Swiss 3T3 cells with or without single or multiple PMT treatments. **A** Representative DNA histograms from a time course of untreated (*a* and *b*) or PMT-treated (*c* and *d*) cells from day 1 (*a* and *c*) and day 4 (*b* and *d*). To the *right* is the summary of the percentages of untreated (*top*) or PMT-treated (*bottom*) cells found in G_0/G_1, S, and G_2/M phases of the cell cycle during the time course. **B** Representative DNA histograms from multiple PMT treatments: (*a*) untreated cells analyzed on day 5; (*b*) cells treated with PMT on day 5 and analyzed on day 6; (*c*) cells treated with PMT on day 0 and analyzed on day 5; (*d*) cells treated with PMT on day 0 and again on day 5 and analyzed on day 6. The percentages of cells found in G_0/G_1, S, and G_2/M phases of the cell cycle are also shown.(Reprinted with permission from Wilson et al. 2000.)

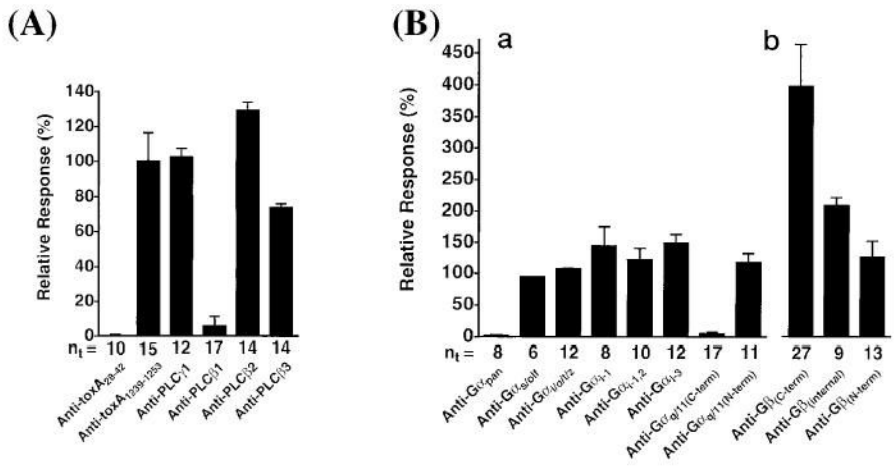

Fig. 2.

Identification of the intracellular target of PMT as $G_{q/11}$ by using specific antibodies against key signaling proteins to block the PMT response in *Xenopus* oocytes. The peak inward Cl^- current was used to measure the effect of specific antibodies on the PMT-induced Ca^{2+}-dependent Cl^- currents in oocytes voltage-clamped at a holding potential of –80 mV. Antibodies against PMT (N-terminus or C-terminus) and various PLC isoforms (**A**) or against various Gα or Gβ subunits (**B**) were microinjected into the oocytes 3 h prior to microinjection with PMT. (Reprinted with permission from Wilson et al. 1997.)

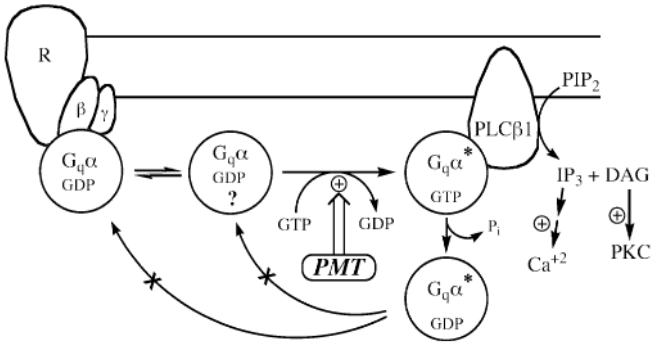

Fig. 3.

A proposed model for PMT action on G_q-coupled PLC signal transduction. In this model, PMT acts on free, monomeric $G\alpha_q$, most likely in the GDP-bound form, and converts it into an active form, presumably GTP-bound, which stimulates PLCβ1. The PLCβ1 hydrolyzes PIP_2 into IP_3 and DAG, leading to Ca^{2+} mobilization that results in the Ca^{2+}-dependent Cl^- current. The PMT-induced response is transient due to GTPase activity of $G\alpha_q$, which is still intact and is stimulated by interaction with PLCβ1. The presumably modified GDP-bound G_q can be neither acted upon again by PMT nor reassociated with the Gβγ-receptor complex.

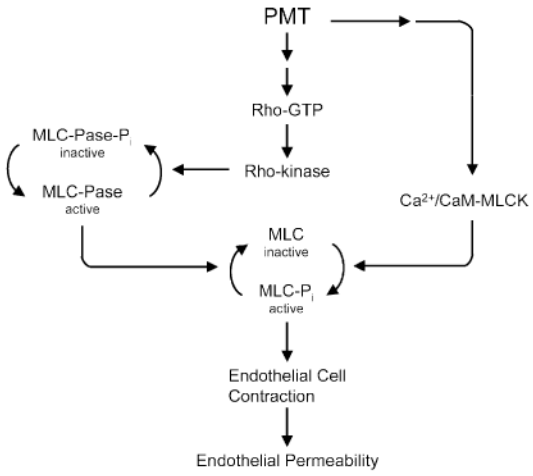

Fig. 4.

A proposed model for PMT action on Rho-dependent vascular permeability. In this model, PMT stimulates the conversion of Rho protein into its active GTP-bound state. Activated Rho then stimulates Rho kinase to phosphorylate and thereby inactivate myosin light chain phosphatase (*MLC-Pase*), which in turn prevents the dephosphorylation of MLC, keeping it in its active phosphorylated state. PMT concomitantly causes the release of Ca^{2+} and activation of Ca^{2+}/calmodulin-dependent MLC kinase (*Ca^{2+}/CaM-MLCK*), which phosphorylates and activates MLC, resulting in endothelial cell contraction and consequent endothelial permeability. (Adapted from Essler et al. 1998.)

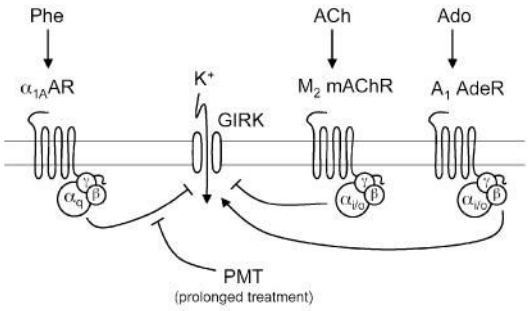

Fig. 5.

A proposed model for PMT action on GIRK channel inhibition by $G_{i/o}$- and G_q-coupled receptors. In this model, opposing sets of G protein-coupled receptors modulate GIRK channels. Stimulation of $G_{i/o}$–coupled receptors, such as A_1 adenosine receptors, M_2 mAChR muscarinic acetylcholine receptors, or serotonin ($5\text{-}HT_{1A}$) receptors, causes the release of Gβγ subunits, which directly interact with and activate GIRK channels to increase K^+ currents. Rapid desensitization of the GIRK channel results from subsequent inhibition via the $G\alpha_{i/o}$ subunits. Inhibition of GIRK currents by the G_q-coupled receptors, such as α_{1A}-AR, ET_AR, M_1/M_3 mAChAR, or thyrotropin-releasing hormone (*TRH*) receptors, involves activation of PLCβ1 by the released $G\alpha_q$ to cause depletion of PIP_2 from the membrane, which results in inactivation of the GIRK channel. Prolonged treatment with PMT uncouples the G_q-signaling pathway and prevents inhibition of GIRK by G_q-coupled receptors, but not desensitization through $G\alpha_{i/o}$. *ACh*, acetylcholine; *Phe*, phenylephrine; *Ado*, adenosine.

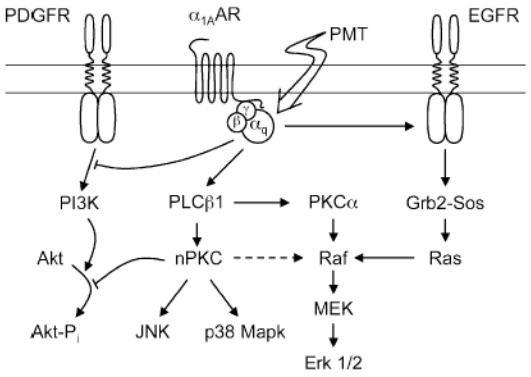

Fig. 6.

A proposed model for PMT action on G_q-mediated mitogenic signaling. In this model, activation of G_q protein by either PMT or G_q-coupled receptors, such as α_{1A}-AR or α-thrombin receptor, results in activation of PLCβ1, as well as transactivation of the EGF receptor via $G\alpha_q$ subunit and inhibition of the PDGF receptor. In HEK-293 cells and cardiac fibroblasts, subsequent activation of the Erk1/2 cascade is mediated predominantly via $G\alpha_q$ transactivation of the Ras-dependent EGF receptor tyrosin kinase pathway, with no significant contribution derived from the PLCβ1-dependent activation of the PKC pathway. In cardiomyocytes, PMT stimulates via the $G\alpha_q$-PLCβ1 pathway both Ca^{2+}-dependent PKCα and novel nPKCs (*PKC*δ and *PKC*ε), which in turn lead to activation of the Erk1/2, as well as p38 MAPK and JNK cascades. PMT both inhibits PDGF receptor-dependent PI 3-kinase (*PI3K*) activation and prevents subsequent activation of the Akt-dependent survival pathway.

Table 1

Signaling of heterotrimeric G-proteins and their modulating toxins

Subfamily	Gα	Signaling[a]	Modulating toxin	Toxin effect on Gα	References[b]
G_i subfamily	G_{i1}	↓AC	PT	Inhibition	(a) (b)
	G_{i2}	↓AC	PT	Inhibition	(a) (b)
	G_{i3}	↓AC	PT	Inhibition	(a) (b)
	G_o	↓Ca^{2+} channels	PT	Inhibition	(a) (b)
	G_t	↑cGMP-PDE	PT	Inhibition	(a) (b) (c)
			CT	Activation	(a) (b) (d)
	G_{gust}	↑PDE	PT	?	(e)
	G_z	↓AC	?	?	(f) (g)
G_s subfamily	G_s	↑AC	CT	Activation	(a) (b)
	G_{olf}	↑AC	CT	Activation	(h)
G_q subfamily	G_q	↑PLCβ, ↑Rho	PMT	Activation/ Inhibition[c]	(i) (j) (k)
	G_{11}	↑PLCβ, ↑Rho	?	?	(k)
	G_{14}	↑PLCβ	?	?	
	G_{15}	↑PLCβ	?	?	
	G_{16}	↑PLCβ	?	?	
G_{12} subfamily	G_{12}	↑Rho	?	?	
	G_{13}	↑Rho	?	?	

[a] AC: adenylate cyclase; PDE: phosphodiesterase; PLC: phopholipase C

[b] (a) (Fields and Casey 1997), (b) (Casey and Gilman 1988), (c) (Van Dop et al. 1984a), (d) (Van Dop et al. 1984b), (e) (Gilbertson et al. 2000), (f) (Casey et al. 1990), (g) (Ho and Wong 2001), (h) (Jones et al. 1990), (i) (Wilson et al. 1997), (j) (Zywietz et al. 2001), (k) (Vogt et al. 2003)

[c] Initial activation, followed by uncoupling of G_q signaling. Prolonged treatment with PMT results in inhibition