

Structure, function, pharmacology, and therapeutic potential of the G protein, $G_{\alpha/q,11}$

Danielle Kamato¹, Lyna Thach¹, Rebekah Bernard¹, Vincent Chan¹, Wenhua Zheng^{2,3}, Harveen Kaur⁴, Margaret Brimble⁴, Narin Osman¹ and Peter J. Little^{1*}

¹ Discipline of Pharmacy, Diabetes Complications Group, School of Medical Sciences, Health Innovations Research Institute, RMIT University, Bundoora, VIC, Australia

² State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Centre, Guangzhou, China

³ Faculty of Health Sciences, University of Macau, Macau, China

⁴ Department of Chemistry, University of Auckland, Auckland, New Zealand

Edited by:

Ian Megson, University of the Highlands and Islands, UK

Reviewed by:

Suowen Xu, University of Rochester, USA

Stephen James Leslie, NHS Highland, UK

*Correspondence:

Peter J. Little, Discipline of Pharmacy, School of Medical Sciences, RMIT University, PO Box 71, Bundoora, VIC 3083, Australia
e-mail: peter.little@rmit.edu.au

G protein coupled receptors (GPCRs) are one of the major classes of cell surface receptors and are associated with a group of G proteins consisting of three subunits termed alpha, beta, and gamma. G proteins are classified into four families according to their α subunit; G_{α_i} , G_{α_s} , $G_{\alpha_{12/13}}$, and G_{α_q} . There are several downstream pathways of G_{α_q} of which the best known is upon activation via guanosine triphosphate (GTP), G_{α_q} activates phospholipase C β , hydrolyzing phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol triphosphate and activating protein kinase C and increasing calcium efflux from the endoplasmic reticulum. Although G proteins, in particular, the $G_{\alpha_{q/11}}$ are central elements in GPCR signaling, their actual roles have not yet been thoroughly investigated. The lack of research of the role on $G_{\alpha_{q/11}}$ in cell biology is partially due to the obscure nature of the available pharmacological agents. YM-254890 is the most useful G_{α_q} -selective inhibitor with antiplatelet, antithrombotic, and thrombolytic effects. YM-254890 inhibits G_{α_q} signaling pathways by preventing the exchange of guanosine diphosphate for GTP. UBO-QIC is a structurally similar compound to YM-254890, which can inhibit platelet aggregation and cause vasorelaxation in rats. Many agents are available for the study of signaling downstream of $G_{\alpha_{q/11}}$. The role of G proteins could potentially represent a novel therapeutic target. This review will explore the range of pharmacological and molecular tools available for the study of the role of $G_{\alpha_{q/11}}$ in GPCR signaling.

Keywords: G proteins, GPCR, cell signaling, therapeutic targets, transactivation

INTRODUCTION

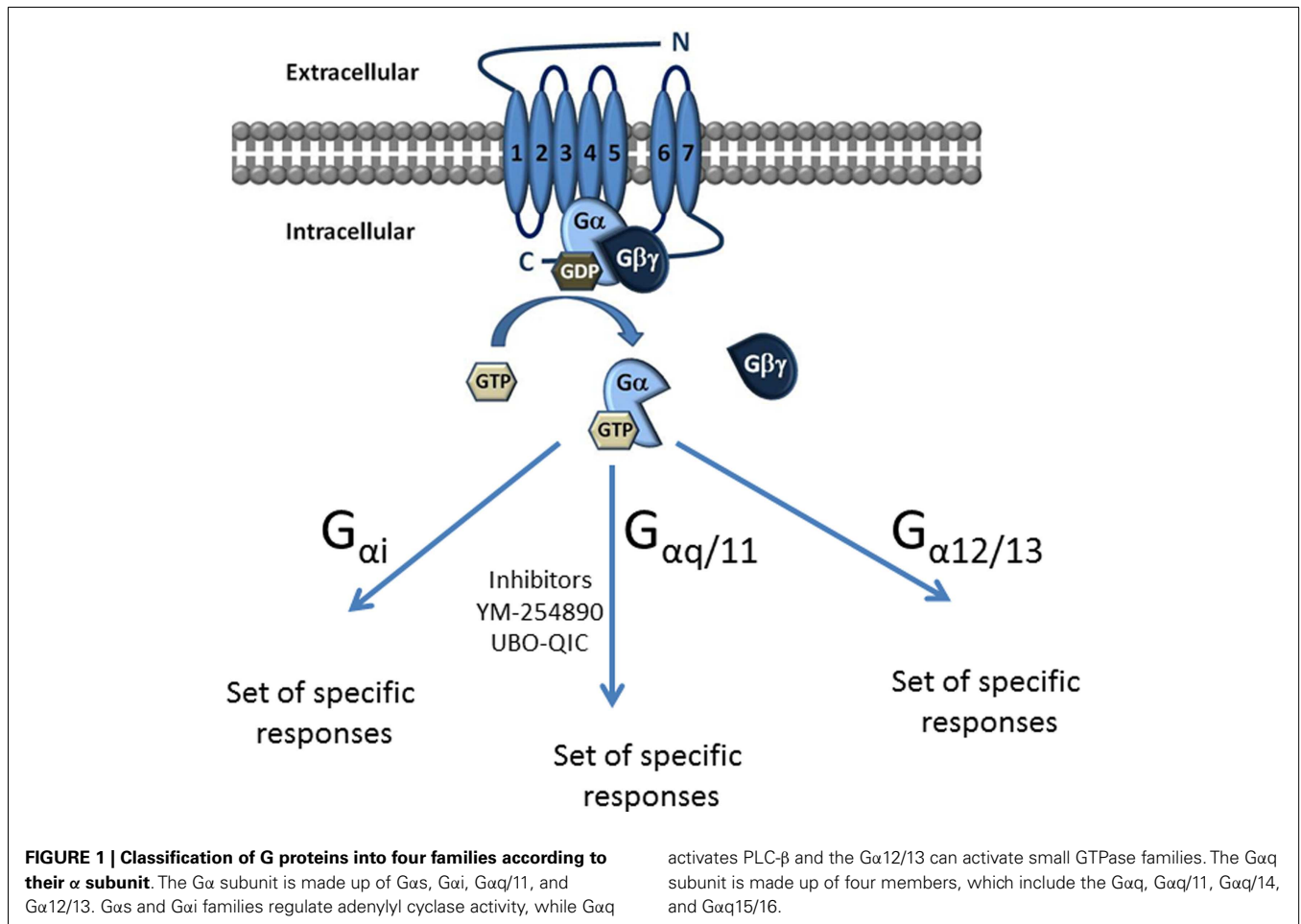
G protein coupled receptors (GPCRs) constitute the largest class of cell surface receptors. GPCR genes account for 5% of the human genome (1, 2). Of these receptors, all are seven membrane spanning receptors but not all are G protein binding but it is convenient to refer to the receptors as GPCRs. GPCRs also represent the largest and among the most efficacious class of therapeutic targets for diseases including cardiovascular disease, cancer, and asthma (1, 2). Many drugs have been developed based on GPCRs and these include some of the most important agents in human medicine, for example, in the treatment of asthma and hypertension (3). GPCRs are helical transmembrane receptors complemented by functional extracellular and intracellular loops (4). Within the GPCR superfamily, there have been five major families identified. They are the rhodopsin, secretin, glutamate, adhesion and frizzled/taste2 families (5). Most GPCRs contain seven helices and three intracellular loops; however, some members of the rhodopsin family may have eight helices and four intracellular loops (6). GPCRs bind hormones, neurotransmitters, or growth factors (7), which initiate a plethora of cellular responses. GPCRs are generally ligand activated but they can also bind to G_{α} -subunits in the absence of a

ligand, a phenomenon known as receptor pre-coupling. GPCRs interact with their respective G proteins only upon receptor activation known as the collision coupling model or in the absence of agonist known as the pre-coupled receptor model (8).

Whereas protein tyrosine and serine/threonine kinase receptors have intrinsic catalytic activity, GPCRs do not have enzymatic activity *per se* but are linked to G_{α} proteins, which are GTPases, and mediate the signal transduction (9). G proteins of the α , β , and γ families provide the specificity and functionality of GPCRs.

G proteins are classified into four families according to their α subunit: G_i , G_s , $G_{12/13}$, and G_q (Figure 1). The G_s and G_i families regulate adenylyl cyclase activity, while G_q activates phospholipase C β and $G_{12/13}$ can activate small GTPase families (10). The G_q family consists of four members: G_q , G_{11} , G_{14} , and $G_{15/16}$ (11, 12) and their respective α subunits are thus G_{α_q} , $G_{\alpha_{11}}$, $G_{\alpha_{14}}$, and $G_{\alpha_{15/16}}$ (Figure 1).

The role of G proteins in GPCR signaling has not been as intensively investigated as other aspects of GPCR signaling possibly due to the limited availability of convenient pharmacological tools. The most useful pharmacological agent has been the compound known as YM-254890, which is a cyclic depsipeptide isolated from



the *Chromobacterium* sp. Initial studies indicated that this is a specific inhibitor of $G\alpha_{q/11}$. YM-254890 has had variable availability and has not been available in recent times. As the importance of GPCR signaling in physiology and pathophysiology continues to grow, the potential importance of G proteins increases both for the fundamental cell biology and as potential therapeutic targets.

One of the major and expanding areas of GPCR signaling is transactivation-dependent signaling (13) in which GPCRs transactivate protein tyrosine kinase (PTK) and protein serine/threonine kinase receptors (14–16). Transactivation greatly expands the roles of GPCRs in cell biology (13, 17–19). GPCR transactivation of PTK receptors was discovered in 1996, has been the subject of almost 200 publications, and has been recently reviewed (20). Our laboratory has recently extended the paradigm of GPCR to PTK receptor transactivation to include the transactivation of protein serine/threonine kinase receptors and specifically the protease-activated receptor (PAR)-1 and endothelin receptor (ETR)-mediated transactivation of the transforming growth factor (TGF)- β type I receptor (TGFBR1) also known as Activin-like Kinase (Alk)-V (15, 16, 21). There is very little information on the role of $G\alpha$ proteins in GPCR transactivation signaling. There is a need for synthetic programs to provide new molecules with the pharmacological properties of YM-254890 and such programs will provide agents, which allow for a much broader range of studies on

the role of G proteins in GPCR signaling. This review focuses on the role of $G\alpha_{q/11}$ in GPCR signaling in the context that the availability of new tools will shortly lead to a large increase in studies in this area. The two targets of compound such as YM-254890 are $G\alpha_q$ and $G\alpha_{11}$ – these two proteins are distinct gene products but they have an identical number of amino acids and essentially identical structures and functions. In this review, we refer to $G\alpha_q$ but most statements will also relate to $G\alpha_{11}$ and only where differences are known and of significance will this distinction be drawn.

$G\alpha_{q/11}$ SIGNALING

The responses to GPCR agonists and the conformational changes in the GPCR that are induced by ligand binding are transduced and then mediated by heterotrimeric G protein complexes. Consisting of three subunits α , β , and γ , their role is to transduce external stimuli into intracellular signaling cascades. Most of the specificity of signaling resides in the $G\alpha$ subunit. In an inactivated state, the α subunit binds guanosine diphosphate (GDP); however, upon binding activation of the GPCR, GTPase activity is induced and promotes the exchange of bound GDP for guanosine triphosphate (GTP). The α subunit and $\beta\gamma$ complex then dissociate from one another and interact with their associated effectors (22). In the most common signaling pathways, $G\alpha_q$ activates phospholipase C β (PLC β), which hydrolyzes phosphatidylinositol

4,5-bisphosphate (PIP₂) releasing diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP₃). DAG activates a number of isoforms of protein kinase C (PKC), whereas IP₃ diffuses to the endoplasmic reticulum (ER) and binds to IP₃ receptors on ligand-gated calcium channels on the surface of the ER leading to a massive release of calcium ions into the cytosol and subsequently in some cells, the opening of cell surface calcium channels leading to the influx of extracellular calcium (23). The calcium cycle continues with the uptake of calcium back into the ER by Ca ATPases.

In addition to this paradigm, it has been shown that RhoA is a mediator of calcium sensitization and is downstream of G_α signaling. Activation of the members of the Rho family is via GTP binding. The exchange of GDP for GTP on these proteins is controlled through guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP (24). Activation of Rho-mediated signaling pathways can be indirectly mediated by GPCRs, integrins, or receptor tyrosine kinases. G proteins, G_α₁₂ and G_α₁₃, activate Rho by activation of a Rho GEF (25). It is only when RhoA is active that it can interact with and activate downstream effectors such as Rho kinase (ROCK).

Thrombin activation of PAR-1 involves both G_α_{q/11} and G_α_{12/13}, which causes RhoA activation signaling downstream to stimulate ROCK and PKC-related kinase. RhoA activation coupled to G_α_{q/11} involves intracellular release of calcium involving the downstream activation of the two Rho-regulated protein kinases, which in turn regulates the contraction of actomyosin and the formation of focal adherence in human endothelial cells. In the case of G_α_{12/13}, RhoA is activated through GEFs such as p115 RhoGEF, PDZ-RhoGEF, or leukemia-associated RhoGEF. In the case of G_α_{q/11}, it is suggested that the GEFs utilized may involve p63 Rho GEF or Trio; however, the specific GEFs involved in this signaling pathway are yet to be confirmed.

Overexpression of active G_α₁₁ or stimulation of the m1 muscarinic acetylcholine receptor induces apoptosis in HeLa cells. Rho kinase and ROCK are stimulated due to the cleavage of activated caspase 3 during apoptosis. There have been several studies on the mechanisms involved in G_α_{q/11}-induced apoptosis, which show that this phenomenon is cell- and context-dependent. In COS-7 and CHO cells, G_α_q-induced apoptosis is dependent on PKC, and angiotensin II-induced myocyte apoptosis is dependent on the release of intracellular calcium suggesting the involvement of PLC pathway. The molecular mechanism of G_α_{q/11} induced apoptosis leading to the activation of Rho/ROCK is not clearly understood; however, some studies have shown that G_α_{q/11} signaling activated RhoA, which inhibited insulin-stimulated Akt phosphorylation in HeLa cells. In CHO cells, G_α_q and G_α₁₁ regulate actin cytoskeleton remodeling through the activation of ADP-ribosylation factor 6. Platelets stimulated with P2Y₁ agonist leads to the activation of RhoA, this activation was inhibited by G_α_q inhibitor YM-254890, indicating that RhoA activation downstream of purinergic (P2Y)-1 receptors requires G_α_q stimulation (26).

STRUCTURE OF G_α_q

G_α_q and G_α₁₁ are distinct gene products but from the same chromosome (12). These two proteins have an identical number of amino acids and are functionally almost identical. However, the tissue distribution of the two isoforms is distinct (12). G_α_q is a 359

amino acid protein comprising two domains: a helical domain and a GTPase binding domain. The GTPase domain is responsible for hydrolyzing GTP to GDP, as well as binding the Gβγ subunits, GPCRs, and other effectors. This domain is conserved between all members of the G protein superfamily (6). The GTPase domain contains three switch regions, which are flexible loops that change conformation when bound with GTP. The helical domain contains six α-helices, which encapsulates nucleotides in the protein core by forming a lid over the nucleotide-binding pocket. Of all G protein families identified, members of the G_α_q family share the most amino acid sequence homology. In humans, G_α₁₁, G_α₁₄, and G_α₁₆ share 90, 80, and 57% sequence similarities, respectively (27).

FUNCTIONS OF G_α_q

G_α_q plays a role in platelet aggregation. Bleeding time and resistance to thromboembolism are dramatically increased in G_α_q-deficient mice compared to wild type (28). G_α_q is also implicated in insulin-stimulated glucose transport (29). In 3T3-L1 adipocytes, G_α_q is required for insulin-induced GLUT4 translocation and the stimulation of 2-deoxy-D-glucose uptake. Angiotensin II dose-dependently increases cell proliferation in smooth muscle cells and this is inhibited by the G_α_q antagonist, GP-2A (30). G_α_{q/11} proteins are involved in HIV-1 envelope glycoprotein-dependent cell-cell fusion upstream of Rac-1 (31). Genetically modified mice studies suggest that receptors coupled to the G_α_q play a role in the development of heart failure (32). Following treatment to activate G_α_q in transgenic mice expressing a silent G_α_q, the mice rapidly developed a dilated cardiomyopathy and heart failure. Transgenic mice expressing an inducible G_α_q that cannot activate PLCβ do not develop heart failure. Thus, the activation of G_α_q resulting in heart failure requires the activation of PLCβ (32).

ROLE OF G_α_q IN THE GPCR TRANSACTIVATION OF KINASE RECEPTORS

There are now two major pathways of GPCR to cell surface receptor kinase transactivation – the well-established transactivation of PTK receptors, notably epidermal growth factor receptor (EGFR) and the recently identified transactivation of serine/threonine kinase receptors, specifically the TGFBR1 (14–16, 33). There is some information of the role of G_α proteins and thus G_α_q in the transactivation of PTK receptors but nothing is known of the role of G_α_q in the transactivation of serine/threonine kinase receptors.

G protein coupled receptors coupled to G_α_q, such as bombesin receptor or G_α_i proteins, such as M2 muscarinic acetylcholine receptor, expressed in COS-7 cells show increased EGFR tyrosine phosphorylation more than that resulting from G_α_i coupled receptor stimulation. Cells transfected with G_α_q-coupled GPCRs are unaffected by pertussis toxin while G_α_i coupled receptors are, as expected, blocked by pertussis toxin treatment (34). Thus, EGFR transactivation may occur through both pertussis toxin-sensitive and -insensitive pathways. GPCR transactivation of serine/threonine kinase receptors and specifically TGFBR1 by both ETR and PAR-1 has been identified in vascular smooth muscle cells (VSMCs) but the role of G_α_q in transactivation of TGFBR1 has not been reported (15, 16, 21).

Thus far, the biochemical mechanisms of GPCR to protein tyrosine and protein serine/threonine kinase receptors have been found to be completely distinct with, for example, the former involving MMPs and the latter being independent of MMPs (16). The transactivation of serine/threonine kinase but not tyrosine kinases involves the cytoskeleton (16). The independent signaling pathways have made it difficult to envisage a single potential therapeutic target for the inhibition of all GPCR transactivation signaling (18).

It will be interesting to investigate the role of G_{αq} proteins in tyrosine and serine/threonine kinase transactivation signaling as it has the potential to be a point of commonality in GPCR-mediated transactivation of cell surface protein tyrosine and serine/threonine kinase receptor signaling.

MOLECULAR AND PHARMACOLOGICAL REGULATION OF G PROTEINS

G proteins in cells can be effectively knocked down utilizing a molecular approach and this has allowed for detailed studies of the function of various G proteins and their interactions. Classic experimental approaches assume that the intervention is specific and does not alter other parameters that would impact on the experimental result of the index intervention. This is not always the situation and is certainly not the reliable paradigm in the case of the regulation of G proteins. Gilman and colleagues (35) demonstrated that knocking down G_β proteins resulted in a compensatory increase in both the effector, adenylyl cyclase and even the GPCR, being the β₂-adrenergic receptor. Results of knock down interventions are also not always reciprocal – the knock down of one G protein may lead to a compensatory increase in another G protein family member but the reverse or reciprocal phenomenon may not occur (35). Thus, the knock down of G_{αq} and G_{α₁₁} in HeLa cells increased the accumulation of G_{α_i} and G_{α₀} but the reciprocal response did not occur (35).

G_α and β proteins exist in approximately equal mass stoichiometry in most cells. This occurs primarily because G_β proteins stabilize bound G_α proteins with the corollary that free G_α proteins are degraded. However, G_α proteins are subject to palmitoylation and myristoylation and these processes may bind G_α proteins to the cell membrane and stabilize the proteins (36). A consequence of the role of post-translational regulation on stability and the cellular levels of G proteins is that the relationship between mRNA and protein levels may be perturbed. Higher mRNA levels may lead to increased expression of the G protein, but if it is orphaned and free the protein may be degraded providing for high level of mRNA and in the presence of low levels of protein.

Molecular approaches to the up- and down-regulation of target proteins are a major component of modern mechanistic studies of cell biology. However, as exemplified above, alteration of target protein levels may result in compensatory changes in other components of a system and the perturbation might not provide the expected result. Pharmacological approaches nullify the activity or function of a target protein without in most cases altering the level of the target protein. If there is greater availability of G protein inhibitors such as YM-254890 or alternative new tools, then it will be interesting to determine if blocking a G_α protein results in

any changes in the level of other G proteins within the cell. Such studies are currently underway in our laboratory.

PHARMACOLOGY OF G_{αq} INHIBITORS

YM-254890

The compound known as YM-254890, a cyclic depsipeptide isolated from the *Chromobacterium* sp. QS3666, is a specific G_{αq} inhibitor. YM-254890 has been shown to inhibit ADP-induced platelet aggregation, which is mediated via GPCRs, P2Y₁, and P2Y₁₂ (37). These receptors are associated with the G_{αq} and G_{α_i} signaling pathways, respectively. YM-254890 has no effect on the P2Y₁₂ signal transduction pathway, indicating that the compound has some specificity for G_{αq}. It was also shown to inhibit G_{αq}-coupled GPCR signaling by inhibiting calcium mobilization in P2Y₂-expressing C6-15 cells but not cAMP accumulation (38).

YM-254890 inhibits the signal transduction of G_{αq} by inhibiting the exchange of GDP for GTP preventing the activation of the G protein, rather than receptor-G_{αq} interactions (38). When bound to GDP, the non-polar side chains of YM-254890 form hydrogen bonds with the Switch I region; however, this is a conformation that cannot be maintained when bound with GTP (39). Aside from antiplatelet activity, by electrically inducing carotid artery thrombosis in rodents, YM-254890 was also shown to have antithrombotic and thrombolytic effects (40).

YM-254890 was discovered and developed by Yamaguichi Pharmaceuticals, Japan; Yamaguichi subsequently became the property of Astellas Pharmaceuticals, Japan. YM-254890 was made available to researchers 10 years ago and a small number of interesting studies were published. The initial results indicated that YM-254890 is a useful tool for investigating G_{αq/11}-coupled receptor signaling and the physiological roles of G_{αq/11}. For example, G_{αq} knockout mice have lower blood pressure than appropriate controls (41). This indicates some potential for a G_{αq} inhibitor to be an anti-hypertensive agent and accordingly YM-254890 has not been provided to researchers presumably because of such identified commercial value. As discussed above, molecular approaches in this area, for example, G protein knock down can lead to rebound increases in other G proteins with unexpected results. Accordingly, it is understood that a number of groups are undertaking programs for the synthesis of compounds related to YM-254890 and it is likely that the availability of potent and specific G_{αq/11} inhibitors would greatly expand activity and knowledge in this area and answer important questions such as the role of G_{αq/11} in GPCR transactivation signaling of protein kinase receptors.

UBO-QIC/FR300359

FR300359, henceforth referred to as UBO-QIC, is also, like YM-254890 a cyclic depsipeptide; it is isolated from the *Ardisia crenata sims* plant (42). UBO-QIC is structurally very similar to YM-254890 and not surprisingly shows similar pharmacological activity. UBO-QIC inhibits platelet aggregation in rabbits *in vitro* and causes dose-related hypotension in anesthetized normotensive rodents, which is consistent with the effect on blood pressure in G_{αq} knock down mice (41, 43). The blood pressure lowering effect was attributed to the ability of UBO-QIC to partially mediate nitric oxide release from endothelial cells and inhibit calcium migration caused by voltage-dependent and receptor-operated channels (44).

Since the discovery of UBO-QIC as a Gα_q antagonist, there have been limited studies showing its use. In HEK cells transfected with TRPV4, PAR-2-mediated intracellular calcium release was abolished by UBO-QIC when compared to control; however, extracellular calcium influx through the TRPV4 ion channel was unaffected thus showing that PAR-2 coupling to TRPV4 is not mediated by Gα_q signaling (45). There have been no studies directly comparing the activity of YM-254890 and UBO-QIC possibly because of the linked variability of the former compound whereas at the time of preparing this review, UBO-QIC is commercially available.

THE PEPTIDE ANTAGONIST GP-2A

In 2004, Tanski et al. (30) discovered a competitive Gα_q inhibitor, G Protein antagonist-2A, also known as GP-2A. GP-2A is a peptide that selectively inhibits the action of Gα_q by M1 muscarinic cholinergic receptors. The signaling pathway of Gα_q and its role in cell proliferation with rat pulmonary artery smooth muscle cells were studied. Angiotensin II-mediated proliferation, PLCβ activation, and Erk1/2 phosphorylation were inhibited by more than 50% in the presence of GP-2A (30). The EGFR can be activated by EGF to generate an intracellular signaling pathway leading to the phosphorylation of several downstream effector proteins such as Erk1/2 (46). Tanski and colleagues have evaluated angiotensin II (as specific Gα_q agonist) to effectively reduce Erk1/2 activation mediated by PLCβ via Gα_q in the presence of GP-2A by showing its association with the phosphorylation of Erk1/2 in rat pulmonary artery smooth muscle cells (30). This study provides a strong foundation for our laboratory research as we can further investigate the

possibility of this downstream signaling pathway to see whether or not GP-2A can act on other GPCR agonists such as thrombin to effectively respond similarly via Gα_q in other smooth muscle cell types such as human VSMCs.

OTHER PHARMACOLOGICAL TOOLS FOR EVALUATING THE ROLE OF Gα_q IN GPCR SIGNALING

It is also possible to indirectly assess the role of Gα_q in GPCR signaling by analyzing downstream events through the use of inhibitors (Figure 2). For example, as detailed above GPCR ligand engagement activates Gα_q which in turn activates phospholipase C leading to the catalysis of PIP₂ and the release of DAG and IP₃. There are inhibitors of PLC-β including U73122, and its inactive analog U-73343 is available to use as a control compound. These compounds have been widely used (47–51) although they are not considered to be especially useful and specific agents. The antibiotic, neomycin, can also be used as a PLCβ inhibitor in that it binds to the target substrate, PIP₂ and inhibits the action of PLCβ to release DAG and IP₃, which can be assessed as a reduction in IP₃ accumulation or increased free intracellular calcium (23). As always with pharmacological approaches, it is likely that the use of multiple approaches can provide the best information on the role of Gα_q in GPCR signaling (Table 1).

U73122 AND ITS INACTIVE ANALOG U-73343

To maximize our knowledge of Gα_q, it is possible to examine the downstream role of Gα_q in GPCR by assessing the inhibitors of PLC, U73122, and its inactive analog U73343. U73122 and its analog U73343 were used to show the effect of human platelet

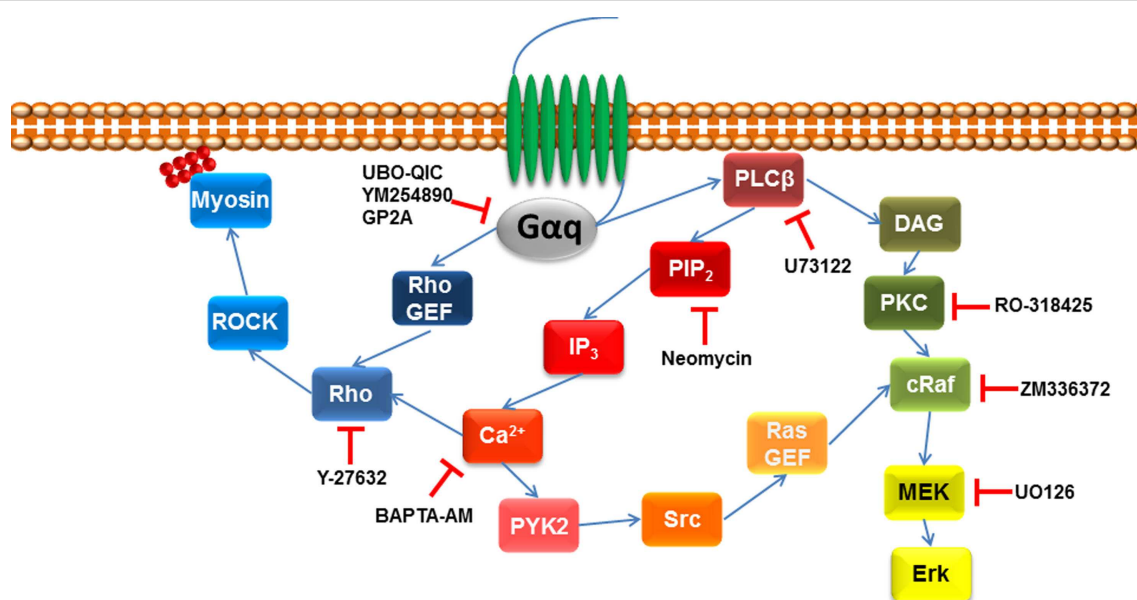


FIGURE 2 | Pharmacological agents to inhibit downstream signaling intermediated of Gα_q. Once GPCR is activated by its agonist, Gα_q signaling activates phospholipase C β (PLCβ), which leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and diacylglycerol (DAG). The former leads to initiate the release of 1,4,5-inositol tris phosphate (IP₃) initiating calcium release, activating protein tyrosine kinase 2 (PYK2), which

leads to proto-oncogene tyrosine protein kinase (Src) activating Ras guanine nucleotide exchange factor (Ras GEF), which leads to the activation of MAPK signaling. MAPK signaling pathway can also be downstream of DAG that activates protein kinase C (PKC), which leads to the activation of MAPK signaling. Gα_q signaling can also go indirect of PLCβ by activating Rho GEF leading to the activation of the Rho/ROCK signaling pathway.

Table 1 | Pharmacological tools used as inhibitors of Gα_q or its downstream signaling intermediates.

	Inhibitors	Actions	Reference
Direct	YM-254890	Gα _q inhibitor by inhibiting the exchange of GDP for GTP	(37) (42)
	UBO-QIC	preventing the activation of the G protein	
	GP-2A	Competitive Gα _q inhibitor	(30)
Downstream	U73122/ U73343	PLC-β inhibitors	(47)
	Neomycin	Binds to PIP ₂ and blocks the action of PLC-β	(23)
	BAPTA-AM	A chelator of calcium ions preventing increase in intracellular calcium	(52)
	Y-27632	RhoA/ROCK specific antagonist	(53)

calcium signaling and protein tyrosine phosphorylation in the presence of thrombin, collagen, and thapsigargin (47). U73122 showed complete inhibition of calcium signaling in the presence of this agonist, which was generated via the activation of PLC specifically the β and γ isoforms (47, 54). U73343 did not show any calcium inhibitory effect via the activation of PLC but rather showed the calcium inhibitory effect via the upstream activation of cPLA2 in the presence of thapsigargin and collagen (47). This provides a clear indication that U73343 has minimal activity as a PLC inhibitor.

The study also investigated the role of Gα_q in the transactivation of PTK receptors to show that platelets, stimulated by thrombin increased protein tyrosine phosphorylation. In the presence of U73122, the phosphorylation of tyrosine kinase was abolished (47). As mentioned earlier, Gα_q are prominent in signaling in VSMCs. From the recent study, it is possible that we can replicate this investigation in other cell type to further study the PTK and perhaps the serine/threonine kinase transactivation pathway in the presence of U73122.

NEOMYCIN AND ITS POTENTIAL ROLE FOR Gα_q SIGNALING STUDIES

As mentioned above, another agent, which can further be investigated, is neomycin, a PLC inhibitor. Our laboratory has previously reported that neomycin strongly inhibits the formation of IP₃ in rat aortic smooth muscle cells in the presence of endothelin, an agonist that influences contraction in smooth muscle (23). Endothelin acts via specific ET-A receptors, which are coupled to PLC to stimulate calcium mobilization (23). ETR is coupled to PLC via G proteins (55) and its activation acts on the cardiac muscle where it binds to ryanodine located on the SR, which releases calcium mobilization within the cardiac muscle cell (56). As known, the signaling pathways of GPCRs through G proteins contribute to various functions in different cell types such as the contraction of blood vessels and are involved in many diseases such as cancer and cardiovascular disease (16, 57). In unpublished data, we have found that neomycin has a dose-dependent

inhibition of thrombin-mediated release of intracellular calcium in human VSMCs. Further investigation is required to understand its pharmacological signaling pathway via Gα_q.

THE ACTION OF INTRACELLULAR CALCIUM ION TO STUDY THE ROLE OF Gα_q IN GPCR SIGNALING

The activities of calcium ions on the inhibitory action of calcium channel blockers and its impact on atherogenesis in the regulation of proteoglycan biosynthesis in human VSMCs was studied via the role of ionomycin and bis (2-amionophenyl) ethyleneglycol-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) (58).

Ionomycin is a calcium ionophore, which elevates intracellular calcium (58). Radioactive sulfate incorporation into proteoglycans was unaffected by ionomycin, providing support that calcium regulation is not involved in proteoglycan synthesis (58).

Similarly, BAPTA-AM, a chelator of calcium ions, which prevents an elevation of intracellular calcium by acting as a calcium buffer (52) had no effect on proteoglycan synthesis (58). Agonists, TGF-β, and ET-1 stimulated BAPTA-AM to decrease sulfate incorporation into proteoglycans. The interpretation therefore concluded that there were no effects on calcium ion stimulation hence intracellular calcium does not play a role in VSMC proteoglycan synthesis (58).

Y-27632 – A RhoA/ROCK INHIBITOR

Y-27632 is a widely used specific inhibitor of RhoA/ROCK family of protein kinases (53). The ROCK family of kinases is involved in Rho-induced formation of actin-stress fibers and focal adhesion as well as the down-regulation of myosin light chain (MLC) phosphatases. Deng et al. (59) examined the role of the PLC calcium pathway and Rho Kinase in PAR-1-mediated CCL2 release. Rho kinase activation is mediated by a Gα_q-PLC-calcium-dependent PKC pathway to release thrombin-mediated CCL2. Thrombin-induced phosphorylation of MLC was inhibited by PLC calcium and calcium-dependent PKC inhibitors. Q94 a PAR-1 selective Gα_q antagonist abolished thrombin-mediated MLC phosphorylation. Subsequent experiments showed that blockade of Rho kinase signaling is not essential for CCL2 protein production but is important in the release of CCL2 from the cell, as thrombin-mediated CCL2 levels are inhibited by Y-27632.

Having provided evidence that cytoskeletal rearrangement is involved in thrombin-mediated transactivation of TGFBR1, ROCK inhibitor Y-27632 was used to study the role of ROCK in the transactivation signaling pathway. Y-27632 inhibited the downstream product of ROCK-phosphor-Ezrin, Radixin, and Moesin. The ROCK inhibitor abolished the thrombin-mediated increase in phosphoSmad2, indicating that Y-27632 inhibits the activity of ROCK and ROCK is involved in the thrombin-mediated transactivation of TGFBR1. To evaluate the role of ROCK signaling in cardiac contractility, hearts were treated with Y-27632. This led to a significant inhibition in the peak of pressure of non-transfected hearts but no reduction in basal contractility in hearts overexpressed with α1A-adrenergic receptor signifying that ROCK pathways play an important physiological role in maintaining baseline contractility (60).

G α GENE KNOCKDOWN USING siRNA

Despite the very large number of GPCRs, there are relatively few studies that have used the potential of G $\alpha_{q/11}$ gene knockdown by siRNA to explore their roles in the signaling cascades. One of the first reported gene knockdown studies of G α proteins was the knockdown of G α_q and G α_{11} gene expression using siRNA in HeLa cells (35). This work demonstrated an absolute requirement of G $\alpha_{q/11}$ to stimulate histamine-mediated phospholipase C activity. Silencing of G α_q or G α_{11} caused indistinguishable phenotypes, loss of half of histamine-stimulated PLC activity, despite the fact that concentrations of G α_{11} exceed those of G α_q by 10-fold. No compensatory increases of either G α_q or G α_{11} were observed following loss of either protein. Loss of G α_q or G α_{11} did cause increased accumulation of G α_i and G α_o (35). A study characterizing the G α subunits required for PAR-1-mediated endothelial cell permeability showed that both G α_q and G α_{11} were necessary for thrombin to increase permeability while the need for G $\alpha_{12/13}$ was less. Both protein subunit families contributed significantly to RhoA activation by thrombin (61). Knockdown of G $\alpha_{q/11}$ in human pulmonary artery smooth muscle cells alters but does not prevent hypoxia-induced mitogenic factor-mediated calcium release demonstrating that G $\alpha_{q/11}$ contributes to hypoxia-induced PLC signaling pathway (62). Using siRNA knockdown of G α_q or G α_s in human prostate epithelial cells, GPCR melatonin receptor MTNR1A has a dual requirement of G α_q and G α_s receptor coupling for effective MTNR1A signal transduction (63). In HEK cells expressing high levels of thyrotropin-releasing hormone receptor 2, knockdown of G $\alpha_{q/11}$ reduces persistent agonist-induced signaling by 82% and suggests that G $\alpha_{q/11}$ is a required component of the activated receptor signaling pathway (64). Clearly, there is considerable scope to use siRNA technology more often as a very useful tool in delineating the importance of G α proteins in GPCR signaling.

G α KNOCKOUT MICE AND G α PROTEIN MUTANTS AND CHIMERAS

Other molecular approaches to investigate the varied roles of G α proteins include generating G α protein knockout mice or overexpressing a variety of G α mutants and chimeras in cell lines and examining their effects in different cellular contexts.

Over the last decade, the most widely used G α knockout model is the G $\alpha_{q/11}$ knockout mouse. G $\alpha_{q/11}$ -deficient fibroblasts from these knockout mice have been used to study a large number of GPCR signaling pathways. These studies have demonstrated that G $\alpha_{q/11}$ -deficient mouse fibroblasts expressing bradykinin B (2) receptor require both G α_i and G $\alpha_{q/11}$ for effective bradykinin-mediated stimulation of the Erk cascade (65). G $\alpha_{q/11}$ knockout mouse fibroblasts expressing GPCR α 1b-adrenoreceptor protein or fusion proteins consisting of the α 1b-adrenoreceptor and wild-type G $\alpha_{q/11}$ or palmitoylation-resistant G $\alpha_{q/11}$ mutants reveal agonist-mediated receptor/G $\alpha_{q/11}$ -coordinated release of the $\beta\gamma$ complex (66). Expressing fusion proteins consisting of the GPCR α 1b-adrenoreceptor with various G α mutants results in altered receptor contact domain residues and enables the identification of key agonist and antagonist receptor contact sites that are necessary for α 1b-adrenoreceptor activation. An aromatic group four amino acids before the carboxy terminus in G $\alpha_{q/11}$ provides maximal

α 1b-adrenoreceptor activation information (67). Mouse embryonic fibroblasts from double knockout G $\alpha_{q/11}$ and β -arrestin mice demonstrated that kisspeptin activation of GPCR 54 regulates the hypothalamic–pituitary–gonadal axis in reproductive function and that GPCR 54 has a co-dependency of both the G $\alpha_{q/11}$ and β -arrestin pathways in a time-dependent manner to regulate Erk and localize pErk to the nucleus for downstream gene expression (68). Knock-in of ETR type A or type B in G $\alpha_{q/11}$ -deficient mice showed differential craniofacial development is based on specific ETR G α_q and G $\alpha_{q/11}$ requirements (69). Clearly, the G $\alpha_{q/11}$ knockout mouse has proven itself a reliable and useful tool in the study of G α protein-mediated signaling.

An alternative approach to exploring G α protein signals has been to co-express G α protein mutants or chimeras in different cell types. G α_q chimeric mutants containing G α_i or G α_o tails co-expressed in COS-7 cells with opioid receptors and stimulated with opioid agonist are insensitive to pertussis toxin catalyzed ADP-ribosylation demonstrating an inability of G α_i or G α_o tails to serve as pertussis toxin substrates (70). Gi-coupled opioid receptors increase G α_q signals as demonstrated by the co-expression of constitutively active G α mutants in COS-7 cells and requires activated phospholipase beta and G $\beta\gamma$ dimers (71).

A considerable number of studies have explored membrane localization of G α proteins in different cellular contexts and described a diversity of requirements. N-terminal sequence-mutated G α proteins expressed in HEK293 cells are unable to localize to the plasma membrane due to their inability to bind to G $\beta\gamma$ or attach myristate and palmitate (72). Mutated G α_q and G α_s proteins deficient in G $\beta\gamma$ -binding and co-expressed with different $\beta(1-5)$ or $\gamma 2/3$ subunits show that G $\beta\gamma$ and G α proteins promote membrane localization of the other and requires palmitoylation (73). Defects in plasma membrane localization of G α_s occur when four N-terminal basic residues are mutated to glutamine; however, mutation of nine basic residues in G α_q is required. G $\beta\gamma$ co-expression partially rescues localization indicating that the characteristics of the N-terminal residues of G α_s and G α_q are critical in membrane localization of these proteins (74). Using co-expressed constitutively active G α_q or G $\alpha_{q/12}$, the activation of ETRs was shown to mediate the binding of G α_q or G $\alpha_{q/12}$ in caveolae to enable the downstream activation of Erk1/2 (75).

G α protein-mediated signaling studies across a wide variety of GPCRs dominate the literature using G α protein mutants or chimeras. Constitutively active G α_q , G $\alpha_{q/12}$, or G $\alpha_{q/13}$ mutants transfected into Jurkat cells co-expressing GPCR muscarinic cholinergic receptor subtypes demonstrated a requirement for G $\alpha_{q/13}$ to activate downstream transcription factor serum response factor. However, the M1 subtype also required G $\alpha_{q/11}$ and calcium when regulator proteins RGS2 and RGS4 were co-transfected that demonstrates a unique pathway for the M1 receptor (76). G $\alpha_{q/11}$ (Y356D) mutation results in altered GPCR α 1b-adrenoreceptor contact domain and abolishes receptor function, however, does not affect ligand binding (67). Studies using constitutively active mutants G α_q (Q209L) and G $\alpha_{q/13}$ (Q226L) demonstrate that G α_q activates rat brain phospholipase D1; however, G $\alpha_{q/13}$ inhibits its activity (77). G α_q deletion mutants were used to demonstrate that G α_q mediates down-regulation of the vesicle-associated GPCR vesicular monoamine transmitter transporter VMAT2 activity in

platelets (78). Expression of a constitutively active $G\alpha_q$ (R183C) mutant inhibited the expression of ezrin–radixin–moesin-binding phosphoprotein 50 and subsequent internalization of GPCR thromboxane A(2) beta receptor independently of PLC and PKC pathways (79). Specific $G\alpha$ peptides and dominant negative $G\alpha$ mutants were used to demonstrate the ability of α -thrombin to activate different effectors via $G\alpha$, $G\beta\gamma$, and $G\alpha_{i2}$, respectively, in Chinese hamster embryonic fibroblasts and thereby regulate the activation of the PI3kinase/Akt pathway (80). Molecular modeling and testing GST–fusion proteins of $G\alpha_q$ mutants–GPCR kinase complexes revealed a critical residue $G\alpha_q$ Pro185 at the interface with GPCR kinase 2 with residues $G\alpha_q$ K77, L78, Q81, and R92 also playing key interactive roles (81). Constitutively active $G\alpha_q$, $G\alpha_{q/12}$, and $G\alpha_{q/13}$ overexpressed in human astrocytoma cells increased agonist-activated thromboxane A2 receptor-mediated IL-6 production while mutated $G\alpha_q$ and $G\alpha_{q/13}$ overexpression blocks IL-6 production (82). Using both constitutively active and dominant negative $G\alpha_q$ subunit expression showed that in neuroblastoma cells $G\alpha_q$ elicits a rapid signal at the plasma membrane (83). Expression of constitutively active $G\alpha_q$ (Q209L) mutant inhibits Ras and the PI3K/Akt pathway; however, minimal effects are seen on the Ras/Raf/MEK/Erk signaling pathway (84). $G\alpha_q$ mutants that cannot bind $G\beta\gamma$ are unable to be stimulated by the mitogenic Pasteurellosis multocida toxin (PMT) demonstrating the requirement of cohesive $G\alpha_q/G\beta\gamma$ signaling for this toxin activation pathway (85). Expressing GTPase-deficient $G\alpha_q$ mutant in the human adrenal cell line H295R depolarizes the two-pore loop potassium channel TASK and thereby increases aldosterone secretion (86). Chimeric G proteins have been used to determine $G\alpha$ responses from orphan GPCRs with unknown $G\alpha$ coupling partners. A luciferase reporter system with a chimera that contains promoter elements that drive Gs, Gq, and G12 signals and another chimera with promoters to drive Gi signals revealed neuromedin U receptor 1 activating Gq, neuromedin U receptor 2 activating Gi, and luteinizing hormone receptor activating Gq and Gs proteins (87).

POTENTIAL OF $G\alpha_q$ AS A THERAPEUTIC TARGET

$G\alpha_q$ as a protein has several functions, which are valuable therapeutically. The GTPase activity, which hydrolyzes bound GTP to GDP, is an enzyme action that can be targeted. The binding of GDP and GTP are potential targets in the same manner in which the ATP binding site is target of many drugs inhibiting kinases (88). The ligand-activated GPCR acts as a GEF, which stimulates the exchange of GDP for GTP on the $G\alpha$ peptide and this could be targeted. Furthermore, the protein contains a switch mechanism and this can be targeted as it is the target of the YM class of inhibitors (39). So, it is both theoretically possible and has been demonstrated that $G\alpha_q$ can be exploited as a drug target.

The consequences of targeting signaling molecules have theoretical limitations based on the role of such targets in normal physiology but also conceptually there may be situations, pathophysiology, in which the activity of $G\alpha_q$ is elevated or enhanced and presents itself as a target. Such situations are common in therapeutics but in most cases can only be established experimentally.

As discussed above, inhibition of $G\alpha_{q/11}$ using YM-254890 has demonstrated anti-platelet aggregation, antithrombotic, and thrombolytic properties in a rat model of carotid artery thrombosis (40). Therefore, compounds that inhibit $G\alpha_{q/11}$ could show enormous potential in the treatment of thrombotic conditions such as thrombotic stroke and myocardial infarction in humans. Additionally, a number of recent studies have also implicated a role for $G\alpha_{q/11}$ in a range of metabolic conditions such as obesity and type 2 diabetes (89, 90). Activation of $G\alpha_q$ results in pronounced increases in blood glucose levels in a mouse model (89), thus, compounds that inhibit $G\alpha_q$ could also show promise as a future treatment option for type 2 diabetes.

In the important cardiovascular context of hypertension, $G\alpha_q$ knockout mice have reduced blood pressure (41) and YM-254890 has demonstrated some anti-hypertensive properties (40). Although there are many effective anti-hypertensive agents currently available, there are also many subjects with medication-resistant hypertension, which does require a niche for new therapies although it is unclear if a $G\alpha_{q/11}$ inhibitor would be suitable to consider for such a niche.

CONCLUSION

G protein coupled receptor signaling is a major area of cell biology and therapeutics. The functioning of the seven transmembrane GPCR has been one of the most intensively studied areas of protein function. GPCRs signal through G proteins of the α and $\beta\gamma$ subtypes where most of the signaling specificity is determined by the $G\alpha$ protein. For the $G\alpha$ protein family, these signaling pathways include the well-known PLC, PKC, and IP3 pathways and the lesser appreciated Rho/ROCK pathway. For multiple reasons, mostly the limited availability of pharmacological agents, which inhibit G protein function, the role of G proteins in GPCR signaling has been severely under-studied relative to the intense activity around the GPCRs. This is true for the $G\alpha_q$ proteins, which are the subject of this review but also for other G proteins. Given the broad involvement of GPCRs in cellular functioning, this is a major deficit in cellular signaling studies and potentially more importantly in the search for new drug targets. The recently expanding area of GPCR signaling is that of transactivation-dependent signaling in which GPCR transactivation of protein tyrosine and protein serine/threonine kinase cell surface receptors enormously expands the range of activities associated with the respective GPCRs. The potential role of G proteins and $G\alpha$ proteins in particular in GPCR transactivation signaling is one very interesting area to be explored. It is likely that there are programs of chemical synthesis underway to synthesize inhibitors of $G\alpha_q$ proteins and these will increase the availability of inhibitors and also with the importance of this area hopefully lead to new studies, which produce a range of agents, some of which may be useful in *in vivo* studies. It is hoped that such studies may provide insights into the potential role of $G\alpha_q$ in disease processes and reveal the extent to which such inhibitors may represent novel therapeutic agents in a range of conditions from cancer to cardiovascular disease.

AUTHOR CONTRIBUTIONS

PL, NO, VC, and WZ conceived the focus of the review, wrote, and edited the paper. HK and MB provided chemical insight about

cyclic depsipeptide. DK, LT, and RB contributed expertise with preparation of the manuscript and the figures.

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