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VERSITA

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G PROTEIN CO-SIGNALING AND CHALLENGES FOR TRANSLATIONAL RESEARCH

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Abstract

The G_q-linked G protein coupled receptors (GPCRs) and their signaling pathways are important clinical targets for the dementia of Alzheimer's disease and cognitive decline with aging. G_q stimulates phospholipase C-β_1 (PLC-β_1) activity, increasing levels of inositol-1, 4, 5-trisphosphate (IP_3) and diacylglycerol, to initiate mobilization of intracellular Ca^{2+} and activation of protein kinase C, respectively. While high concentrations of ligand typically evoke large sustained increases in cytosolic Ca^{2+} levels, it has long been appreciated that the dynamics of the Ca^{2+} increase are more complex and consistent with multiple levels of regulation. Physiologically relevant concentrations of G_q-ligands evoke rhythmic fluctuations or an oscillation in the level of cytosolic Ca^{2+}. Downstream targets are tuned to respond to the frequency of the Ca^{2+} oscillations which in turn, reflect the oscillations in IP_3 levels. Oscillatory behavior depends on the assembly of self-organizing interactions. The components that contribute to and regulate the Ca^{2+} oscillator have been unclear, precluding transfer of this fundamental knowledge from bench to bedside. Many GPCRs that signal with G_q also co-signal with G_{12}. G protein co-signaling could therefore regulate the Ca^{2+} oscillator. This letter explores the potential relationship between Ca^{2+} oscillations, G protein co-signaling and cellular response in the context of our recent observations. We found that G_q efficacy is synergistic with phosphatidic acid, (PA), a signaling mediator generated downstream of activated G_{12} and RhoA. Regulation by PA depends on interaction with the unique PLC-β_1 PA binding region. G protein co-signaling is therefore a mechanism for GPCRs to collectively assemble self-organizing interactions that regulate the Ca^{2+} oscillator.

Keywords

• Ca^{2+} oscillations • G protein co-signaling • G_q • G_{12} • GPCR • Phospholipase D
• Phosphatidic acid • Phospholipase C-β • Protein kinase C • Diacylglycerol kinase

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1. Introduction

GPCRs (G protein coupled receptors) are widely distributed in the CNS where they regulate critical functions by signaling with their G proteins. In response to the ligand-activated GPCR, G_q engages second messenger systems that control multiple CNS activities, including information processing, learning and cognition [1-3]. G_q stimulates PLC-β lipase activity to increase levels of inositol-1, 4, 5-trisphosphate (IP_3) and thereby mobilize the release of Ca^{2+} from intracellular stores. The increase in diacylglycerol (DAG), with or without an increase in Ca^{2+} levels, stimulates protein kinase C (PKC) activity [4]. Hypo-signaling by the G_q-linked M_1 muscarinic acetylcholine receptor has been linked to the dementia of Alzheimer's disease [5]. GPCRs continue to be the major focus of most CNS drugs [6]. Greater efficacy, selectivity and safety may come from a better understanding of the processes that determine response to activated G_q.

Cellular response to the ligand-activated GPCR has generally been scrutinized in the context of a GPCR working through a specific G protein subtype and its effector [7,8]. This relationship does not adequately describe the signaling dynamics that generally take place in activated cells. The pattern of increase in cytosolic levels of Ca^{2+} by G_q-linked ligands for example is known to be complex and consistent with multiple levels of regulation. Physiologically relevant concentrations of Ca^{2+} mobilizing ligand evoke repetitive Ca^{2+} transients or oscillations [9-11]. This oscillatory response contrasts with the large and sustained increase in cytosolic Ca^{2+} concentration that is generally studied at high doses of ligand. The frequency of Ca^{2+} oscillations has been shown to be regulated by the ligand concentration. Both the identity of the ligand and cell-specific regulatory processes have also been found to shape the oscillations. Downstream targets appear to be tuned to the frequency of the oscillation. G proteins therefore digitalize the Ca^{2+} signal, converting analog information (ligand concentration) to a frequency encoded message.

The conversion of the Ca^{2+} signal to a digital format has important consequences for signal transduction. The sharp threshold for response, characteristic of digital signaling, ensures that leaky activation is prevented [10]. Spurious responses do not occur. A digital signal also has high resolution and stability, enabling transmission across long distances. How the Ca^{2+} oscillator is assembled and regulated however has remained unclear, preventing the transfer of this fundamental knowledge from bench to clinic.

Many GPCRs that signal with G_q generally co-signal with G_{12}. Co-signaling could therefore

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potentially assemble and regulate the Ca^{2+} oscillator. Some GPCRs have been shown to co-signal with all four G proteins subtypes to regulate response [12,13].

This letter will explore the potential relationship between the Ca^{2+} oscillator, G protein co-signaling and response in the context of our recent observations. We found that G_q efficacy is regulated by phosphatidic acid (PA), a signaling mediator generated downstream of activated G_{12} and RhoA [14,15]. We propose that G protein co-signaling is a fundamental mechanism that regulates global response. G protein co-signaling allows a GPCR to assemble and dynamically regulate the interactions that form the Ca^{2+} oscillator. GPCR co-signaling is a potentially high impact clinical target.

1.1 The signaling landscape

The molecular basis for ligand-induced oscillations in levels of cytosolic Ca^{2+} has been the subject of much modeling and discussion. PLC-β lipase activity is a strong candidate mechanism. Oscillations in G_q regulated PLC-β lipase activity could regulate the Ca^{2+} transients. Support for this hypothesis comes from the observation that agonist induced oscillations in cytosolic Ca^{2+} levels depended on oscillations in the levels of IP_3 [16-18]. The levels of DAG [19] and activity of PKC [20,21] were also shown to oscillate. The concurrent rhythmic behavior of DAG is consistent with oscillations in the rate of IP_3 synthesis rather than metabolism.

Oscillations are thought to arise from the formation of stable self-organizing structures that are kinetically distinct from background signaling events [22]. Processes deemed essential for oscillatory behavior include synergism and inter-play between feed-back mechanisms. Synergism is necessary to attain the threshold for stimulation [10]. The frequency of oscillations is determined by the interaction between non-linear positive and negative feedback mechanisms.

Our observation that G_q efficacy is synergistic with PA, a signaling mediator generated downstream of activated G_{12} and RhoA [14,15] may provide a missing piece to this puzzle. G protein co-signaling is a mechanism that could bring together multiple interactions that collectively assemble to regulate oscillations in PLC-β_1 lipase activity (Figure 1). Oscillations in PLC-β_1 lipase activity are generated and sustained by the synergism and feed-back mechanisms controlled by a GPCR co-signaling with G_q and G_{12} and possibly other G proteins. Rhythmic behavior in lipase activity in turn shapes oscillations in the levels of IP_3, Ca^{2+}, DAG and the activity of PKC. The greater the ligand concentration, the higher the frequency of oscillations and impact on the signaling landscape. Dynamic regulation by co-signaling allows the GPCR to adjust the frequency and therefore cellular response as determined by cell-dependent regulation.

2. GPCR-G_q signaling and novel functions of PLC-β

In the current model for G protein activation, the ligand activated GPCR initiates signal transduction by acting as a guanine nucleotide exchange factor (GEF) on the G protein GTPase cycle. The exchange of GTP for GDP on the $G\alpha_{GDP}$ subunit of the G protein heterotrimer is markedly accelerated, increasing the level of the active $G\alpha_{GTP}$ species. The affinity of Gα for GPCR and Gβγ is reduced in its GTP-bound state, resulting in an uncoupling of regulation from the receptor and dissociation of the G protein heterotrimer. Liberated $G\alpha_{GTP}$ and Gβγ subunits engage effectors to regulate the cellular signaling network.

Duration of the activated state depends on the intrinsic Gα GTPase activity which hydrolyzes the bound GTP to GDP. $G\alpha_{GDP}$ re-associates with Gβγ. The heterotrimer re-engages with the receptor. Gα GTPase activity of the G_q and G_i subfamily is increased by GTPase activating proteins (GAPs). GAPs thereby control signaling dynamics. GAPs include the regulators of G protein signaling (RGS), G-protein regulated kinases and PLC-β [23].

GPCRs that couple to the G_q subfamily of heterotrimeric G proteins stimulate phospholipase C-β (PLC-β) lipase activity to increase levels of cytosolic Ca^{2+} and DAG. G_{12} activates monomeric RhoGTPases through the enhancement of RhoGEF activity. G_i signals through Gβγ subunits to regulate multiple effectors, including the PLC-β family. G_s stimulates adenylyl cyclase activity to increase levels of cyclic AMP [7,8].

This convenient classification blurs when attempting to predict effector response to GPCRs that co-signal with multiple G protein subtypes [12]. GPCRs that signal via G_q can co-signal with G_{12}. Some GPCRs co-signal with all four G protein subtypes. The lack of an identifiable conserved sequence in GPCRs that determines selectivity for the G protein subtype offers the possibility that their interaction is regulated. GPCRs that have been shown to co-signal with multiple G proteins include many which contribute significantly to CNS function,

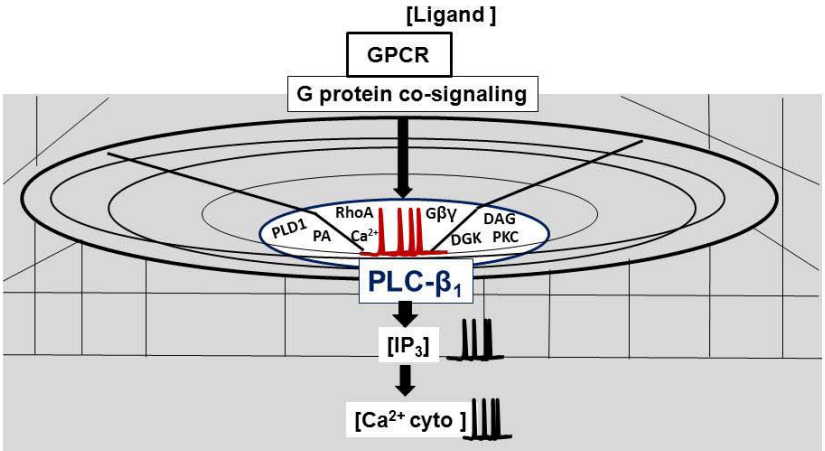

Figure 1. G protein co-signaling is a mechanism to assemble interactions that collectively regulate oscillations in PLC-β_1 lipase activity. Oscillations in PLC-β_1 lipase activity in turn regulate oscillations in levels of IP_3 that determine oscillation in cytosolic Ca^{2+} concentration.

including M_1 and M_3 muscarinic acetylcholine receptors, metabotrophic glutamate 1a receptors and protease activated receptor [12].

2.1 PLC-β and G_q

The four G_q regulated PLC-β isoforms, PLC-β_{1-4}, are distinguished from the other members of the large family of phosphatidylinositol-4,5-bisphosphate (PIP_2)-specific PLCs, by the presence of a long C-terminal tail that contains the residues essential for interaction with key regulators [4]. Differences in the PLC-β isoforms with respect to sensitivity to stimulation by G protein subunits, catalytic activity, regulation by kinases and tissue distribution contribute to define their unique signaling roles. PLC-β may shape response through its many roles as a lipase downstream of $G\alpha_q$, a GAP and binding partner for $G\alpha_q$.

The lipase activity of the PLC-β_{1-4} isoforms is stimulated by $G\alpha_q$. PLC-β_{1-3} but not PLC-β_4 is also stimulated by Gβγ subunits, derived principally from activation of the pertussis-toxin sensitive G_i. Residues that mediate high affinity interaction with $G\alpha_q$, necessary for stimulation of PLC-β lipase activity and the increase in cytosolic Ca^{2+} levels, have been mapped to the distal region of the PLC-β_1 C-terminal tail [24,25]. The PLC-β GAP domain lies adjacent to and partially overlaps with residues necessary for $G\alpha_q$ stimulated lipase activity.

A novel role for PLC-β GAP in regulating signaling dynamics has recently been proposed [26]. The dual function of PLC-β as both a GAP and effector for $G\alpha_q$ can actually result in an increase in GPCR signaling efficiency through kinetic scaffolding. The general view holds that GAPs deactivate signaling [23]. The GAP-mediated increase in $G\alpha_q$ GTPase activity has been proposed to kinetically scaffold with GPCR GEF activity. The interaction between GPCR, $G\alpha_q$ and PLC-β is kinetically stabilized. This three protein complex, GPCR, G_q and PLC-β_1, was found to be resilient to dissociation over several GTPase cycles [27]. PLC-β GAP activity may therefore permit the ligand-activated GPCR to retain control of G_q signaling.

Finally, PLC-β may also competitively regulate how $G\alpha_q$ interacts with its large family of binding partners. $G\alpha_q$ interacts dynamically with PLC-β_{1-4}, RGS proteins, G protein regulated kinase 2 (GRK2), p63RhoGEF and phosphatidylinositol-3 kinase. GRK2, like PLC-β, is both an effector for $G\alpha_q$ and a GAP for $G\alpha_q$. GRK2 could therefore also regulate GPCR signaling efficiency. Altering the interaction between $G\alpha_q$ and its many binding partners could alter signaling kinetics and physiological outcome [28].

The PLC-β_1 and PLC-β_3 isoforms are highly expressed in the telencephalon and cerebellum, respectively [29,30]. Disruption in the murine PLC-β_1 gene resulted in epileptic seizures [31], abnormal anxiety profiles and memory impairment [32]. Loss in human PLC-β_1 expression has been reported and found to be associated with early-onset epileptic encephalopathy [33]. The extent that the observed phenotypes reflect disruption in the function of PLC-β_1 as a lipase, GAP or binding partner for $G\alpha_q$ has not been evaluated. Appropriate avenues of treatment depend on understanding the relationship between phenotype and the multiple functions of PLC-β.

2.2 Synergism with phosphatidic acid

We demonstrated that $G\alpha_q$ stimulated PLC-β_1 lipase activity was synergistic with PA, a phospholipid mediator generated downstream of the G_{12} and RhoA-regulated phospholipase D1 (PLD1) in transfected COS-7 cells [14,15]. Regulation by PA required a unique PLC- β_1 PA-binding motif that mapped to a defined region within the C-terminal tail [15,34]. Neither basic nor hydrophobic residues were essential for regulation by PA. Stimulation by PA was enhanced by titration with Ca^{2+}, as determined by *in vitro* studies using purified proteins [35]. The significance of the potentiative stimulation by Ca^{2+} remains to be determined. Ca^{2+} may participate in a positive feedback mechanism to augment response to PA.

Disruption in regulation by PA occurred with the replacement of a single residue within the PLC-β_1 PA-binding motif. A 10-fold decrease in agonist potency and 60% decline in maximum stimulation by the activated G_q-linked M_1 muscarinic acetylcholine receptor was observed [14]. Residues essential to stimulation of PLC-β_1 activity by PA did not overlap with those required for stimulation by the G protein subunits, $G\alpha_q$ or Gβγ, or for membrane association [34]. Regulation by PA therefore occurred through a dedicated binding domain.

The PLC-β_1 PA binding region was found to be conserved across mammalian species but not present in the other PLC-β isoforms. That different PLC-β PA-binding regions may contribute uniquely to regulation of activity is suggested by the observation that PLC-β_3 lipase activity was also stimulated by PA, albeit with a lower sensitivity than PLC-β_1 [36]. Whether this difference in sensitivity to PA reflects isoform specific regulation by different cellular sources of PA is not known at this time. The answer requires that we identify the PLC-β_3 PA-binding domain. An intriguing possibility is that G protein co-signaling via PLC-β_1 or PLC-β_3 may distinctly shape the frequency of the Ca^{2+} oscillation to target specific downstream pathways.

2.3 Phosphatidic acid

Levels of PA increase rapidly in response to the ligand-stimulated increase in PLD activity [37]. Diacyglycerol kinases (DGK) also produce PA [38] and the role of this family of kinases in regulation will be discussed latter. The two major mammalian PLD isoforms, PLD1 and PLD2, were found to differ in their regulation and coupling to intracellular signaling networks. PLD1 but not PLD2 activity was stimulated by the G_{12} activated RhoA. Only the PLD1 isoform has been linked to synergism with $G\alpha_q$ stimulation as dependent on the PLC-β_1 PA binding region [15]. The extent that PLD1 and PLD2 may co-ordinate to regulate stimulation of PLC-β_1 (and perhaps PLC-β_3) will be important to determine. The G_{12} subtype consists of two members, $G\alpha_{12}$ and $G\alpha_{13}$, which can uniquely regulate cellular response [8]. Which member may contribute to regulation by co-signaling has not been determined.

In addition to PLC-β_1, PA has been shown to regulate a broad spectrum of targets that include lipases, kinases and GAPs [39]. A consensus sequence for regulation has not been identified but regulation generally appears to depend on a short linear sequence. The PA-binding domain therefore differs from the defined globular structures that have been shown to bind phosphoinositides [40]. Unlike PLC-β_1, many but not all targets depend on electrostatic interactions for regulation by PA

[39], as may be important for mediating their translocation or association with membranes. PA is an anionic phospholipid. Dependence on the PA concentration for regulation also varies considerably across targets.

The novelty of PA as a mediator of signaling is further illustrated by the observation that a basic PA-binding sequence from one protein was found to be ineffective in mediating regulation in a related protein [41]. PA mediates translocation of Raf kinase to the membrane in response to activated Ras. The binding site for PA was localized to a basic sequence within the Raf-1 kinase domain. The basic amino acid motif in the Raf kinase PA-binding domain is conserved in the kinase domain of Arabidopsis CTR1, a plant homologue of Raf1. This sequence however was ineffective in mediating regulation of CTR1 by PA. The PA-binding region localized to a different linear sequence within the CTR1 kinase domain that lacked basic residues necessary for electrostatic interactions.

2.4 Phospholipase C-ε

G_{12} mediates RhoA-dependent stimulation of PLC-ε lipase activity [42]. PLC-ε is additionally regulated downstream of G_i through Gβγ and G_s through exchange protein activated by cyclic AMP (Epac). PA was also shown to stimulate PLC-ε activity [43]. PLC-ε represents another candidate effector for regulation by G protein co-signaling.

PLC-ε was found to be associated with the process of neuronal differentiation but high levels of PLC-ε expression continued to persist after differentiation [44]. While the function of PLC-ε in neurons remains unclear, it is possible that an increase in PLC-ε lipase activity could generate a pattern of Ca^{2+} oscillations that differs from PLC-β. In Rat-1 fibroblasts for example, the agonist stimulated increase in PLC-$β_3$ and PLC-ε lipase activity was found to occur in a temporally distinct manner [45].

3. Deactivation by PKC

PKC has been shown to participate in negative-feedback regulation of Ca^{2+} oscillations but mechanisms have remained unclear [10]. PKC-mediated disruption in the synergism for stimulation of PLC-β activity is a novel mechanism that could deactivate the Ca^{2+} oscillator. PKC was found to inhibit stimulation of PLC-$β_1$ lipase activity by Ca^{2+} [46], Gβγ [47] and PA [36]. PA synergizes with $Gα_q$ to stimulate PLC-$β_1$ lipase activity [36]. Stimulation of the PLC-$β_3$ isoform by $Gα_q$ and by Gβγ was also shown to be inhibited by PKC [48]. $Gα_q$ and Gβγ synergize to stimulate PLC-$β_3$ lipase activity [49].

PKC constitutes a large family of lipid-regulated serine-threonine kinases, activated downstream of G_q signaling. A hallmark of PKC behavior is that they regulate response through localized signaling [50,51]. The PKC family consists of 10 members, grouped into 3 major classes by their regulatory domains. The C1 and C2 regulatory domains determine the recruitment/activation of PKC isoforms to the membrane in response to the increase in levels of intracellular mediators. Stimulation of conventional PKCs (α, βI, βII, γ) requires an increase in the levels of both Ca^{2+} and DAG. The increase in cytosolic Ca^{2+} concentration promotes rapid translocation to the membrane via the C2 domain. At the membrane, DAG increases cPKC activity and also retains cPKC at the membrane, allowing for greater signaling efficiency. The C1 domain in the novel PKCs (δ, ε, θ, η) has a higher sensitivity to DAG but the C2 domain is relatively insensitive to Ca^{2+}. Activation of nPKC occurs independent of an increase in Ca^{2+} levels. The atypical PKC (ζ, ι) depends primarily on protein-protein interaction for activation.

The localized control of signaling is thought to depend on the coupling of PKC activity to oscillations in cytosolic Ca^{2+} levels [51]. In astrocytes, glutamate stimulation induced a rapid oscillation in cPKC translocation that was dependent on oscillations in both the levels of DAG and Ca^{2+} [20]. The cPKC activity, as measured by phosphorylation of a membrane associated reporter substrate, was found to oscillate, lagging a few seconds behind the Ca^{2+} oscillations [21].

The localized increase in Ca^{2+} and DAG levels also appeared to restrict the translocation of cPKC to specific regions on the membrane in response to ligand [19,52]. *In vitro* studies show that the fatty acyl composition of DAG can determine stimulation of PKC activity [53]. Different species of DAG may therefore further refine translocation of PKC to unique membrane domains.

3.1 PKC as a negative feedback regulator of PLC-β lipase activity

PKCα inhibited PA and Gβγ stimulation of PLC-$β_1$ lipase activity, as was inversely related to their concentration [36,47]. Inhibition by PKCα was specific as stimulation by activated $Gα_q$ was unimpaired. Inhibition of PLC-β activity by PKC may occur independent of kinase activity, through a novel protein: protein interaction. PKCα has been shown to regulate target proteins independent of kinase activity. The regulatory domain of PKCα was found to be sufficient for stimulation of PLD1 activity [54].

3.2 Negative feedback regulation of PLC-$β_3$ by PKC (and by Protein Kinase A)

PLC-$β_3$ lipase activity was found to be inhibited by PKC through both a kinase-dependent and independent mechanism. Inhibition of $Gα_q$-stimulated activity was phosphorylation-dependent but inhibition of Gβγ stimulation occurred independent of phosphorylation [48]. The cPKC, PKCβI and PKCγ, were both shown to phosphorylate PLC-$β_3$ at Ser^{1105}. The PKC isoform that mediates phosphorylation-independent inhibition of PLC-$β_3$ was not identified. Whether unique PKC isoforms contribute in the negative feedback regulation of PLC-$β_1$ and PLC-$β_3$ activity is not known.

PLC-$β_3$ lipase activity was also similarly inhibited by protein kinase A (PKA) [55]. Inhibition of $Gα_q$ but not Gβγ stimulation required phosphorylation at Ser^{1105} *in vitro*. PKA is activated downstream of G_s signaling and the subsequent increase in cyclic AMP levels. GPCR co-signaling at the level of PLC-$β_3$ may contribute to uniquely regulate Ca^{2+} oscillations.

3.3 PKC as a negative regulator of novel PLC-β functions

We do not know whether PKC and PKA may also alter under-investigated novel functions of PLC-β. Is regulation by these kinases restricted to inhibition of PLC-β lipase activity? Disruption in PLC-β GAP activity by kinases

could decrease GPCR signaling efficiency. Altering the affinity between PLC-β and $G\alpha_q$ could modify signaling through other G_q binding partners, affecting the kinetics and dynamics of the response.

4. Diacylglycerol kinases in the regeneration of signaling

DGKs are novel candidate proteins for mediating the recovery of Ca^{2+} oscillations from the PKC-inhibited state. DGKζ was recently shown to be a positive regulator of G_q efficacy in transfected COS-7 cells [56]. Similar to PKC, DGKs are a superfamily of serine threonine kinases that regulate response through localized control of signaling [38]. DGKs phosphorylate the localized increase in PLC-generated DAG to PA. DGKs could therefore initiate recovery by restraining PKC activity and producing the mediator, PA.

The 10 DGK isoforms have been grouped into five subtypes based on their regulatory domains: Type I (α, β, γ), Type II (δ, η, κ), Type III (ε), Type IV (ζ, ι) and Type V (θ) [38]. Type I DGKs have Ca^{2+} binding EF domains that make them sensitive to an increase in Ca^{2+} levels. The ubiquitously expressed DGKζ has a myristoylated alanine-rich C-kinase substrate domain (MARCKS). DGKε lacks regulatory domains but is responsible for resynthesis of the PIP_2 substrate. DGKs are cytosolic and translocate to the membrane in response to stimulation by ligand.

4.1 DGKs disrupt negative feedback regulation by PKC in a cell-dependent manner

Genetic depletion of DGKζ resulted in a dramatic decrease in carbachol stimulated PLC-β lipase activity in transfected COS-7 cells, as mediated via the G_q-linked M_1 muscarinic acetylcholine receptor [56]. Regulation of PLC-β_1 activity is inhibited by PKCα [36,46,47]. Unrestrained negative feedback regulation by PKCα in DGKζ-depleted cells was proposed to account for the decrease in lipase stimulation.

Regulation of DGKζ activity has been shown to be cell-dependent making it difficult to predict impact on PKC activity. The DGKζ MARCKs domain was phosphorylated by PKCα in HEK293 cells [57]. Phosphorylation by PKCα inhibited DGKζ activity and resulted in sustained DAG-signaling. Phosphorylation however was also shown to increase DGKζ activity. In other cells DGKζ is phosphorylated and activated by extracellular signal-regulated kinase (ERK) [58].

4.2 Stimulation by DGK-generated PA

PA derived from DGK activity and the RhoA-regulated PLD1 may distinctly regulate G_q signaling. DGKs mediate localized increases in PA levels and are coupled to the increase in DAG levels by PLC-β lipase activity. The G_{12}-RhoA-PLD1-dependent increase in PA levels occurs in parallel with G_q stimulation. Differences in spatial and temporal characteristics may therefore allow the separate pools of PA to contribute uniquely to outcome.

Consistent with this hypothesis, pharmacological inhibition of PLD activity did not fully recapitulate the phenotype of the selectively PA-impaired PLC-β_1 mutant [14]. Inhibition of PLD activity with primary alcohol decreased agonist potency but did not alter maximum response to carbachol in transfected COS-7 cells. In contrast, disruption in the PLC-β_1 PA binding region by mutagenesis resulted in a marked decrease in both agonist potency and maximum stimulation.

A possible explanation of these results is that the PA generated by the RhoA-regulated PLD1 functions to lower the threshold for ligand stimulation, thereby regulating potency (Figure 2). The subsequent increase in DGK-generated PA levels determines maximum response, both by deactivating PKC inhibition and by stimulating PLC-β_1 activity through synergism. These interactions may further depend on the affinity state of the PLC-β_1 PA-binding domain [34] and cell -specific regulation of DGK activity.

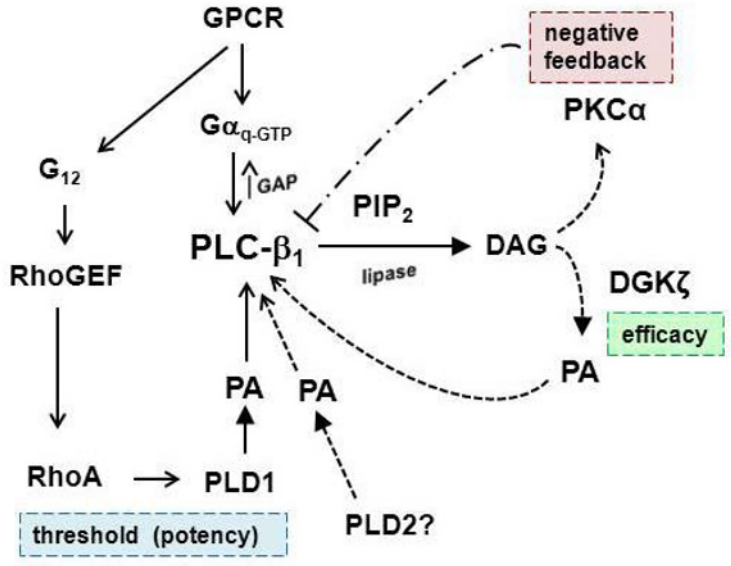

Figure 2. PA generated by two spatially and temporally distinct pathways may uniquely regulate threshold (potency) and efficacy as dependent on the unique PLC-β_1 PA-binding motif. PA derived from the G_{12}-RhoA stimulated PLD1 synergizes with activated $G\alpha_q$ to decrease the threshold (increase potency) for agonist stimulation of PLC-β_1 lipase activity. Synergism with PA requires binding to and signaling via the unique PLC-β_1 PA binding motif. The PLC-β_1 generated DAG regulates response efficacy through PKC and DGK. DAG stimulates PKCα which functions in a localized negative feedback loop to inhibit signaling at the level of PLC-β_1. Negative feedback regulation by PKCα is tempered by DGKζ which converts DAG to PA. The DGKζ generated PA synergizes with activated $G\alpha_q$ to regenerate PLC-β_1 lipase activity, as dependent on the PLC-β_1 PA binding motif. The extent that regulation by different sources of PA may depend on novel functions of PLC-β_1 as a GAP and binding partner for $G\alpha_q$ is not known. Contribution of PLD2 to regulation and the identity of the relevant member of the G_{12} subfamily has not been determined.

4.3 Co-signaling by G_q and G_{12} may assemble a self-organizing domain

How G protein co-signaling could organize multiple interactions that create and regulate the Ca^{2+} oscillator is depicted in Figure 3. PA generated downstream of activated G_{12} synergizes with activated $G\alpha_q$ to lower the threshold for ligand stimulation of PLC-β_1 lipase activity, as dependent on the unique PLC-β_1 PA binding domain. The function of PLC-β_1 to enhance signaling efficiency through kinetic scafolding is also augmented. The synthesis of IP_3 stimulates the release of Ca^{2+} from intracellular stores. The increase in cytosolic Ca^{2+} concentration acts in a positive- feedback loop to amplify lipase activity. Ca^{2+} however also co-ordinates with DAG to initiate negative feedback regulation of PLC-β_1 activity by PKC. PKC deactivates lipase activity (and GAP activity?) through the disruption of synergism between $G\alpha_q$ and PA. Lipase activity is regenerated by the activation of DGKζ which restrains the action of PKCα and generates PA.

The balance between synergism and feedback mechanisms sustains and regulates the frequency of oscillation in PLC-β lipase activity, IP_3 and Ca^{2+} levels. The localized increase in signaling lipids dynamically adjusts the kinetics and frequency of oscillations through allosteric regulation. Signaling phospholipids also scaffold their targets to the membrane, stabilizing the structure against diffusion. Cell-dependent regulation and co-signaling with different PLC isoforms (PLC-β_3 or PLC-ε) may further shape the frequency of the Ca^{2+} oscillations, allowing for different cell types to regulate specific downstream targets in response to activated GPCR.

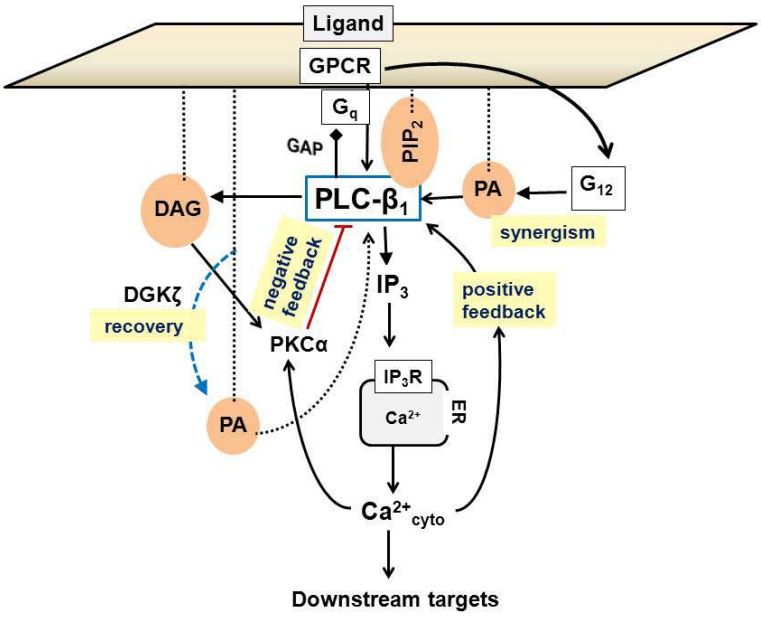

Figure 3. G protein co-signaling could bring together multiple interactions that self-organize to regulate oscillations in PLC-β_1 lipase activity and levels of cytosolic Ca^{2+}. The G_{12}-RhoA -PLD1 generated PA synergizes with activated $G\alpha_q$ to lower the threshold for stimulation of PLC-β_1 lipase activity, as dependent on the PLC-β_1 PA –binding motif. PLC-β hydrolyzes its substrate, phosphatidylinositol -4,5-bisphosphate (PIP_2), to increase the levels of IP_3 and DAG. The binding of IP_3 to the IP_3 receptor (IP_3R) in the endoplasmic reticulum (ER) causes the release of Ca^{2+} from intracellular stores and an increase in the levels of cytosolic Ca^{2+} (Ca^{2+}_{cyto}). Ca^{2+}_{cyto} mediates positive feedback regulation of signaling, synergizing with PA and $G\alpha_q$, to amplify production of IP_3 and thereby increase Ca^{2+} release. Ca^{2+}_{cyto} also contributes to deactivate signaling by co-ordinating with DAG to stimulate PKCα activity. PKCα exerts localized negative feedback regulation of PLC-β_1 activity by disrupting the synergism between $G\alpha_q$, PA and Ca^{2+}. The resultant decrease in synthesis of IP_3 leads to a drop in the levels of IP_3 and Ca^{2+}_{cyto}. IP_3 is metabolized and Ca^{2+}_{cyto} is re-sequestered to the ER and/or pumped out of the cell. These actions of PKC however are attenuated by DGKζ which converts DAG to PA. Regeneration of IP_3 and Ca^{2+}_{cyto} levels occurs due to recovery of regulation by synergism. In this way the frequency of the oscillations in IP_3 and Ca^{2+}_{cyto} levels is regulated by the co-ordinated interplay between PKCα, DGKζ, G_{12}, RhoA, PLD and G_q as mediated by PA and dependent on the PLC-β_1 PA binding motif. This multiprotein complex is stabilized by kinetic scaffolding and anchoring by signaling lipids, PIP_2, PA and DAG.

5. Conclusions and Future

A major challenge, facing translational neuroscience, has been to accurately predict clinical outcome based on leads developed at the laboratory bench. This is due in part to signaling complexity. Signaling by activated G proteins is a dynamic process that can proceed via multiple downstream targets, depend on the genetic background of the cell and on the identity of the ligand. Screening assays which assess multiple responses are therefore being employed to generate an activity profile for ligands that could predict clinical outcome. Key activities however may be missed resulting in a drug with low efficacy and/or unexpected toxicity. Cell-specific regulatory processes in native cells may further thwart the predicted clinical result.

Another approach could be to read the information encoded in the Ca^{2+} oscillator. One might then selectively alter response by targeting the Ca^{2+} oscillator or predict the clinical response by reading a drug's signature on the frequency of Ca^{2+} oscillations. Target proteins appear tuned to the frequency of the Ca^{2+} oscillations [10].

A better understanding of how G protein co-signaling may regulate the Ca^{2+} oscillator is an important first step to achieving these important goals. Studies in this laboratory have identified interactions that could self-organize to form the Ca^{2+} oscillator during G protein co-signaling in transfected cells. Extending these studies to native neuronal cells and evaluating the impact of the various isoforms (PLC-β, PKC, PLD, DGK, G_{12}, PLC-ε), cell-specific genetic background and ligand in regulating the signature of the Ca^{2+} oscillator is an approach to decode the message and transfer this fundamental knowledge to the clinic.

Conflict of Interest

None

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