Synergistic Activation of Phospholipase C-β3 by Gαq and Gβγ Describes a Simple Two-State Coincidence Detector

Finly Philip, Ganesh Kadamur, Rosa González Silos, Jimmy Woodson, and Elliott M. Ross

Summary

Background: Receptors that couple to Gi and Gq often interact synergistically in cells to elicit cytosolic Ca2+ transients that are several-fold higher than the sum of those driven by each receptor alone. Such synergism is commonly assumed to be complex, requiring regulatory interaction between components, multiple pathways, or multiple states of the target protein.

Results: We show that cellular Gα2-Gq synergism derives from direct supra-additive stimulation of phospholipase C-β3 (PLC-β3) by G protein subunits Gβγ and Gαq, the relevant components of the Gi and Gq signaling pathways. No additional pathway or proteins are required. Synergism is quantitatively explained by the classical and simple two-state (inactive-active) allosteric mechanism. We show generally that synergistic activation of a two-state enzyme reflects enhanced conversion to the active state when both ligands are bound, not merely the enhancement of ligand affinity predicted by positive cooperativity. The two-state mechanism also explains why synergism is unique to PLC-β3 among the four PLC-β isoforms and, in general, why one enzyme may respond synergistically to two activators while another does not. Expression of synergism demands that an enzyme display low basal activity in the absence of ligand and becomes significant only when basal activity is ≤ 0.1% of maximal.

Conclusions: Synergism can be explained by a simple and general mechanism, and such a mechanism sets parameters for its occurrence. Any two-state enzyme is predicted to respond synergistically to multiple activating ligands if, but only if, its basal activity is strongly suppressed.

Introduction

Cells integrate multiple incoming signals, and a response to one signal can depend upon the presence or intensity of others. Most often, acute responses to multiple signals are simply additive, either positively or negatively. Occasionally, however, the response to simultaneous stimuli is markedly greater than the sum of the responses to each stimulus alone. Such superadditive responses may be quantitatively modest, but marked synergism can essentially create a Boolean AND gate, or coincidence detector, with which a cell responds significantly only when two signals are present simultaneously. Superadditive responses are not frequent. In a recent large-scale screen for signaling interactions in macrophages, only about 1.5% of the ligand pairs that were tested displayed significant synergism [1]. In some cases, mechanisms of cellular synergism are well understood. These include multiple phosphorylation events, coactivation by transcription factors, induction of synthesis of subsequently regulated proteins, etc. Positively cooperative binding of activating ligands can also create apparent synergism over a narrow range of concentrations as each ligand increases the affinity of the other [2–4]. Scaffolding proteins and membrane surfaces potentiate signals essentially by this mechanism [5–7]. For many acute superadditive cellular responses, however, mechanisms of synergism involve multiple signaling pathways, are otherwise complex [8, 9], or are unknown.

Here we use phospholipase C-β3 (PLC-β3) to elucidate general mechanisms for creating synergism through allosteric regulation, and we show that PLC-β3 regulation accounts for a well-known set of superadditive responses in diverse cells. It has been known for about 15 years that many animal cells and primary cell lines display synergistic Ca2+ responses to simultaneous inputs from different G protein-coupled receptors [10–19]. In these cells, synergism serves as a coincidence detector, such that a robust Ca2+ response and downstream physiological regulation are only observed when both G protein pathways are activated. Such synergism is physiologically important in platelets, neurons, and macrophages [10, 13, 14, 16] and is suggested to play a role in stimulation of mitogenesis in multiple cell types [20]. In most of these cases, one of the two receptors activates Gαi and the other activates Gαq, and synergism does not depend on which Gα or Gα-Gq-coupled receptor initiates the signals. Gαi and Gαq both activate PLC-β isoforms, and the PLC reaction product, inositol-trisphosphate (IP3), triggers Ca2+ release from the endoplasmic reticulum to the cytosol [21]. Gαi stimulates PLC-β via its Gαq subunit, and Gαq acts via its Gβγ subunit [21]. Several studies suggested that the mechanism of synergistic Ca2+ signaling directly involves PLC activation [10, 12, 16–19, 22–24], and recent studies in macrophages and a macrophage-like cell line argue that synergistic stimulation of Ca2+ signaling primarily requires the PLC-β3 isofrom [10]. However, other work suggested that cellular Gαi-Gαq synergism involves interaction between the G proteins [25] or the IP3 receptor [26], and its biochemical mechanism remained unknown.

We show here that purified PLC-β3 responds synergistically to stimulation by Gαq and Gβγ. Synergistic activation of PLC-β3 can exceed ten times the sum of the responses to the individual G protein subunits. Gβγ-Gαq synergism on PLC-β3 can thus quantitatively account for synergistic Ca2+ responses to Gαi and Gαq in cells, and its biochemical behavior is qualitatively consistent with cellular events. Additional proteins or pathways are not required. We also show that the synergistic response of PLC-β3 to Gαq and Gβγ can be explained quantitatively by a simple and classical two-state allosteric model. Synergism does not merely reflect positively cooperative effects of each subunit on the binding affinity of the other, but results from increased accumulation of the active form of PLC-β3. Synergism occurs...
The synergism ratio, the ratio of activities in the presence of both \( G \) (black triangles), 0.2 nM \( G \) synergism ratios at each \( Ca^{2+} \) concentration. The ratio at zero \( Ca^{2+} \) is not accurate because of relative errors in assaying such low activities. The range of

In many cells, simultaneous stimulation of receptors coupled to \( Gi \) and \( Gq \) produces a cytosolic \( Ca^{2+} \) transient that is much larger than the sum of those elicited by the individual receptors. The \( Ca^{2+} \) signal presumably results from \( Ca^{2+} \) release from endoplasmic reticulum, which is triggered by IP3 that is produced by the activity of PLC-b. To see whether the synergistic \( Ca^{2+} \) response in cells reflects direct synergistic activation of PLC-b by \( Gi \) and \( Gq \), we measured the activity of purified PLC-b at increasing concentrations of \( GTP \)-activated \( Gq \) and in the presence or absence of \( Gi \). Together, \( Gq \) and \( Gi \) stimulated PLC-b to an activity nearly ten times the sum of the activities elicited by the two subunits added separately. We define “synergism” generally by this ratio: the activity of an enzyme or signaling pathway in the presence of two regulatory ligands (a and b) divided by the sum of the activities elicited by each ligand (a or b) alone (Equation 1).

\[
\text{Synergism} = \frac{\text{Act}_{ab}}{\text{Act}_a + \text{Act}_b}
\]

If two activities are merely additive, the ratio will be 1.0. Synergism is described by a ratio substantially above 1, and ratios above 10 approach an intuitive definition of coincidence detection.

By this definition, synergism between \( Gq \) and \( Gi \) occurred over a wide range of \( Gq \) concentrations, from 0.03 nM to 9 nM, which approaches saturation. The extent of direct \( Gq \)-\( Gi \) synergism on PLC-b can thus readily account for the 2- to 6-fold synergistic responses of cellular IP3-Ca2+ pathways that have been described for simultaneous stimulation by \( Gq \) and \( Gi \)-coupled receptors.

Superadditive stimulation of PLC-b by \( Gi \) and \( Gq \) also resembles cellular \( Gq-Gq \) synergism qualitatively. \( Gi \) mediates

**Results**

**Gq and Gi Stimulate PLC-b Superadditively**

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Synergism ratios were determined at 0.2 nM GTP to terminate signaling after GTP hydrolysis [21, 27]. Similarly, Gα2-GTP blocked both stimulation of PLC-β3 by Gβγ and its potentiation of Gαq (Table 1). Multiple Gβγ dimers yield superadditive stimulation when added with Gαq (see Table S1, available online), consistent with the occurrence of synergistic responses in diverse cell types. Other experiments used only Gβγ-2. Gβγ-Gαq synergism also requires activation of Gαq by GTP or a nonhydrolyzable analog (GTPγS); Gαq-GDP neither stimulates PLC-β3 nor potentiates stimulation by Gβγ at the highest concentrations tested (Table S2). Hence, all other experiments shown here used Gαq that has been activated by GTPγS. Because Gαq activated by GTPγS or GTP binds Gβγ with relatively low affinity [28], Gβγ does not block its stimulation of PLC-β3.

Gαq-Gβγ synergism was independent of Ca2+ concentration from well below that of resting cytosol (30 pM) to higher than usually reported for stimulated cells (10 μM) (Figure 1B). Responses to Gαq and Gβγ should therefore be potentiable continuously during a cytosolic Ca2+ transient. Ca2+ also had a negligible effect on the EC50 or Hill coefficient for either Gαq or Gβγ, covering almost a 600-fold range of activities (Figure 3). These data were fit to an equilibrium equation (Equation 2) that describes the model of Figure 2. It defines PLC activity as the product of its maximal intrinsic-specific activity, Z, and the fraction of PLC in the four active species at or near the active site, movement of an autoinhibitory structure, altered interaction with the membrane surface, some other event, or a combination of such changes. We used a combination of fitting to experimental data and numerical simulation to ask whether the allosteric mechanism can quantitatively account for both the individual and the synergistic activation of PLC-β3 by Gαq and Gβγ. The activity of PLC-β3 was measured over a wide range of concentrations of activated Gαq and Gβγ, covering almost a 600-fold range of activities (Figure 3). These data were fit to an equilibrium equation (Equation 2) that describes the model of Figure 2. It defines PLC activity as the product of its maximal intrinsic-specific activity, Z, and the fraction of PLC in the four active species shown in Figure 2. The numerator sums each active species and the denominator sums all species. Although this equation is long, it contains few free parameters: binding constants for Gαq and Gβγ (defined for the less active state); an equilibrium constant J that describes the inactive-active conformational equilibrium in the absence of ligand; and two bias constants, F and G, that describe the preference for P* over P described by the bias constants F and G.

**Table 1. Gαq-GDP Blocks Gβγ-Gαq Synergism**

<table>
<thead>
<tr>
<th>[Gαq-GTPγS] (nM)</th>
<th>[Gβγ] (nM)</th>
<th>[Gαq-GDP] (nM)</th>
<th>Synergism Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>10</td>
<td>0</td>
<td>4.6</td>
</tr>
<tr>
<td>0.2</td>
<td>10</td>
<td>30</td>
<td>0.59</td>
</tr>
<tr>
<td>0.2</td>
<td>10</td>
<td>30; heated</td>
<td>4.6</td>
</tr>
<tr>
<td>0.2</td>
<td>5</td>
<td>0</td>
<td>4.7</td>
</tr>
<tr>
<td>0.2</td>
<td>5</td>
<td>15</td>
<td>0.91</td>
</tr>
<tr>
<td>0.2</td>
<td>5</td>
<td>15; heated</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Synergism ratios were determined at 0.2 nM GTPγS-activated Gαq and two concentrations of Gβγ, with or without a 3-fold molar excess of GDP-bound Gαq. Controls contained Gαq that had been heated at 50°C for 60 min. Results show means from two experiments, each with triplicate determinations, and are representative of two additional experiments that did not contain the heated Gαq control. Gαq-GDP also blocked stimulation by Gβγ alone (not shown).

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binding to the more active conformer. Equation 2 assumes that the less active P state has zero activity, which is approximately correct because maximal activity is more than 500-fold above basal:

\[
\text{Activity} = \frac{J \cdot J \cdot F \cdot K_q \cdot |G_{BG}| + J \cdot G \cdot K_q \cdot |G_{BG}^{\beta \gamma}| + J \cdot G \cdot K_q \cdot |G_{BG}^{\beta \gamma}| + F \cdot K_q \cdot |G_{BG}| + 1 + K_q \cdot |G_{BG}| + K_q \cdot |G_{BG}^{\beta \gamma}| + K_q \cdot |G_{BG}^{\beta \gamma}|}{J \cdot J \cdot F \cdot K_q \cdot |G_{BG}| + J \cdot G \cdot K_q \cdot |G_{BG}^{\beta \gamma}| + J \cdot G \cdot K_q \cdot |G_{BG}^{\beta \gamma}| + F \cdot K_q \cdot |G_{BG}| + 1 + K_q \cdot |G_{BG}| + K_q \cdot |G_{BG}^{\beta \gamma}| + K_q \cdot |G_{BG}^{\beta \gamma}|}
\]

(2)

The response of PLC-β3 to a matrix of concentrations of G_{α2} and G_{βγ} was well fit by the allosteric model. Values of constants displayed tolerable statistical errors (Table 2), and overlay of the model-based simulation on the experimental data was clear throughout the ranges of G_{α2} and G_{βγ} concentration (Figures 3 and 4). Values of maximum activities, EC_{50}, and Hill coefficient were all approximated well (Figure 3). Qualitatively similar fits were obtained for two additional similar experiments (not shown). Experimental data are thus consistent with the simple two-state model. To corroborate the values for J, F, and G, we also estimated them from activities measured in the presence of a single high concentration of G_{α2}, G_{βγ}, or both (Table 2). This method is independent of K_{α2}, K_{βγ}, and Z. Values for J and G were similar to those derived from fitting the complete matrix of activities; the value of F was somewhat higher but does not change maximal predicted activation by G_{α2} because even the lower value predicts substantial activation.

The data of Figure 3 and Table 2 indicate that PLC-β3 resides ~99.9% in the inactive state in the absence of G protein under these assay conditions. (Fractional basal activity = J / (1 + J).) Saturating G_{α2} stimulates ~250-fold and saturating G_{βγ} stimulates about 50-fold. Combination of saturating G_{α2} and G_{βγ} together produced about 80% of theoretical total activation (~600-fold) (Table 2). Each subunit thus markedly potentiated PLC-β3 activation by the other. G_{βγ} and G_{α2} also each decreased the EC_{50} of the other (Figure S1), indicating that each G protein subunit reciprocally increases the other’s affinity for PLC-β3. Based on the parameters of Table 2, each subunit increases the affinity of the other about 19-fold, representing ΔΔG ~ 1.8 Kcal for the binding interaction. Such positively cooperative binding is also predicted by the basic allosteric model, which was developed to describe effects on ligand affinity [2, 3]. Note, however, that synergism does not merely reflect the reciprocal increase in the affinity of each subunit by the other. Synergism is above 7-fold at saturating concentrations of G_{βγ} and remains above 2-fold at the highest concentrations of both subunits.

The extent and concentration dependence of G_{α2}-G_{βγ} synergism also agree well with simulation based on the allosteric model (Figure 4), and comparison of data and simulation point out general aspects of allosteric synergism. The synergism ratio displays a pronounced peak at intermediate concentrations of both G_{βγ} and G_{α2}, with a peak value of 10. The ratio falls off at high G_{α2} concentrations but is significantly greater than 2.0 even at saturating concentrations of G_{α2} and G_{βγ} and remains above 1.0 at very low concentrations where activation is minimal. The G_{βγ} concentration did not have a marked effect on the maximally synergistic concentration of G_{α2}, nor did G_{α2} alter the maximally synergistic concentration of G_{βγ}. In all of these aspects, the model-based simulation quantitatively mirrored the experimental data. The two-state allosteric model can thus account for both independent and synergistic regulation of PLC-β3 at steady-state.
If the simple model of Figure 2 quantitatively explains synergistic stimulation of PLC-β
Allosteric Enzyme? What Determines Synergism for a Two-State, Multiactivator isoforms. thus consistent with the cellular behavior reported for the other ratio never significantly exceeded 1.0. This negative finding is
b
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receptors in macrophages, even though the four PLC-
phiq was always additive or less than additive for these three isoforms (examples in Figures S2 and S3). The synergism ratio never significantly exceeded 1.0. This negative finding is thus consistent with the cellular behavior reported for the other isoforms.

Other PLC-β Isoforms Do Not Display Gαq-Gβγ Synergism Seaman and coworkers [10] reported that only the PLC-β3 isoform produces synergistic responses to Gα and Gβγ-coupled receptors in macrophages, even though the four PLC-β isoforms are structurally homologous and PLC-β1, -β2, and -β3 are all individually stimulated by both Gαq and Gβγ. We surveyed activation of PLC-β1, PLC-β2, and PLC-β4 over a wide range of concentrations of both subunits and under diverse assay conditions but found that stimulation by Gβγ and Gαq was always additive or less than additive for these three isoforms (examples in Figures S2 and S3). The synergism ratio never significantly exceeded 1.0. This negative finding is thus consistent with the cellular behavior reported for the other isoforms.

What Determines Synergism for a Two-State, Multiactivator Allosteric Enzyme? If the simple model of Figure 2 quantitatively explains synergistic stimulation of PLC-β3 by Gαq and Gβγ, why do the closely related PLC-β1 and PLC-β2 isoforms not give a synergistic response? More generally, when will an enzyme that is stimulated by noncovalent binding of two or more activating ligands display a synergistic response? How is synergism determined by the parameters of the model?

The simulations in Figure 5 show that the intrinsic isomerization constant J determines both the maximal synergism that can be attained by a two-state allosteric protein and the sensitivity of synergism to the two bias constants F and G. Decreasing J increases synergism, and maximum attainable synergism is approximately inversely proportional to J (Figure 5E). For an enzyme with more than 1% intrinsic activity without ligand (J ≥ 0.01), maximal synergism is at most 2.4-fold (Figure 5B). Sensitivity to the values of F and G is also very sharp, such that only perfectly matched F and G can yield even slight synergism. J = 0.01 is thus the practical upper limit for synergism.

At J = 0.001, about that of PLC-β3, maximal synergism is increased to 10-fold, and the dependences on F and G are far less strict (Figure 5C). Further, synergism is at least 3-fold for almost all reasonable F-G combinations, similar to the behavior of PLC-β3. Thus enzymes that respond to two ligands will display significant potentiative responses if J < 0.001. For lower values of J, maximal synergism increases and dependence on F and G broadens, such that J = 0.0001 can produce > 25-fold synergism over a wide range of F and G (Figure 5D).

Within the limiting maximal synergism that is determined by J, superadditive responses by a given enzyme also depend on the bias constants (Figure 5) and on the concentrations of the ligands relative to their intrinsic affinities for the target enzyme (Figure 4). These two parameters are linked: the dependence of synergism on ligand concentration varies with the bias constants F and G at any fixed value of J (Figure 4 and

<table>
<thead>
<tr>
<th>Table 2. Allosteric Model Parameters for PLC-β3</th>
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<tbody>
<tr>
<td>Matrix Fit</td>
</tr>
<tr>
<td>Z</td>
</tr>
<tr>
<td>J</td>
</tr>
<tr>
<td>Kq</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>Kb</td>
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<tr>
<td>G</td>
</tr>
</tbody>
</table>

Values for the parameters of the allosteric model (Figure 2) were estimated in two ways. Matrix fit parameters (± standard error) were obtained by fitting data from the experiment shown in Figure 3, which was performed at 60 nM Ca²⁺. The complete experiment contained additional data points that were included to improve the quality of the fit based on the results of pilot experiments. Z is the maximum specific activity of the PLC under these assay conditions and varies among assays according to the preparation of phospholipid substrate vesicles. 4-point fit parameters (average of three experiments, ± SD) were calculated from activities obtained at saturating values of Gαq and Gβγ, both, or neither. The 4-point fit is independent of Z, Kq, and Kb. Details are in the Supplemental Information.
When F and G are both high, the synergism ratio displays a sharp dependence on ligand concentrations. When both F and G are decreased, synergism is displayed over a broad concentration range. Thus, for a given enzyme with a suitable value of J, synergism is more likely for two ligands that stimulate with bias constants on the order of 1/J. Further, when the bias constant for only one ligand is high, its optimum concentration is tightly defined but a wide range of concentrations of the weaker activator can promote synergism.

Similarly, the synergism depends less on the precise values of F and G if the concentrations of the two activating ligands are both low (Figure S5). Lower concentrations allow
synergism over a wide range of F and G, but saturating concentrations of both ligands will produce superadditive responses only for a limited range of F and G values. This is the situation for PLC-β3 (Figure 4). In all cases, however, J is the primary determinant of whether synergism will be observed, its maximal extent, and the range of ligand concentrations over which it occurs.

Why PLC-β1, -β2, and -β4 Do Not Respond Synergistically to Gαq and Gβγ

The two-state allosteric model also allows us to explain why only PLC-β3 of the four PLC-β isoforms responds synergistically to inputs from Gα, and Gαq. PLC-β2 responds well to both Gβγ and Gαq. Its behavior was well fit by Equation 2 (Figure S3), consistent with the two-state model, but the values for the constants were strikingly different than those for PLC-β3 (Table S3). Most important, the value of J was 0.15, which precludes synergism (Figure S5). The basal activity of PLC-β2 is 140 ± 45 min⁻¹ under our assay conditions (six duplicate assays), almost 20 times that of PLC-β3. Thus, PLC-β2 fails to display synergism because its basal activity is too high, placing a lid on any possible synergism. In the case of PLC-β1, basal activity is low enough to permit synergism, with J \( \leq 0.003 \), but PLC-β1 is not sufficiently sensitive to activation by Gβγ, PLC-β1 is stimulated less than 4-fold by Gβγ over a wide range of Ca²⁺ concentrations, and it is known to be less sensitive to Gβγ than are the -β2 and -β3 isoforms [21]. For G < 4, simulations do not predict any synergism regardless of Gβγ and Gαq concentrations, even for J \( \sim 0.001 \) (Figure 5, Figure S5). We saw no response of PLC-β4 to Gβγ, as reported previously [29]. Therefore, G < 2 for PLC-β4, similarly disallowing Gαq-Gβγ synergism. The unique ability of PLC-β3 to respond synergistically to Gαq and Gβγ, even though the other PLC-β isoforms do not, is thus explained by the two-state model and the values of the isomerization and bias constants for each enzyme.

Discussion

Synergistic responses to multiple stimuli are relatively rare in biology, but they are important because they allow cells to respond distinctively to two simultaneous signals with novel behaviors. Depending on the dynamics of the signaling pathway, these novel behaviors can take several forms. If each input elicits a minimal response alone and only simultaneous stimulation generates an intracellular signal, then synergism creates a coincidence detector, or logical “AND” gate. Each signal is permissive for the other. Alternatively, each input may be strong enough to initiate signaling on its own, and synergism conveys information on context; each signal is amplified if the other input is present. Such mutual potentiation can be quantitative, more of the same cellular signal, but such amplification can initiate qualitatively new outputs depending on the response thresholds of downstream proteins.

Gαq-Gαq Synergism

This study shows that synergistic signaling by Gα and Gαq-coupled receptors can be explained by the superadditive response of PLC-β3 to stimulation by Gβγ and Gαq. Gα-Gαq synergism has been recognized for over 15 years and is a physiologically important coincidence detector in diverse cells [10, 13, 14, 16, 20]. In cells, Gα provides the Gβγ, because a relatively high Gβγ concentration is required (Figures 3 and 4) and only the Gα family heterotrimers are expressed at high enough levels and release their Gβγ adequately [21, 30]. Gαs are the primary source of Gβγ for all signaling events, apparently for this reason [30].

The 10-fold superadditive response of PLC-β3 to Gβγ and Gαq is quantitatively more than adequate to account for cellular Gα-Gαq synergism over the range of cytosolic Ca²⁺ concentrations. Only PLC-β3 among the PLC-β isoforms displays this behavior, which agrees with the finding that only PLC-β3 permits Gα-Gαq synergism in cells [10]. PLC-β3 is thus a sensitive cellular coincidence detector, one of few allosteric proteins that can act in this way. Gα-Gαq synergism requires no other cellular proteins or pathways. By expression of this isoform, cells can switch between an additive response to Gα and Gαq and a coincidence detection mode.

Synergism demands that both Gαq and Gβγ bind simultaneously to nonoverlapping sites on PLC-β3, as suggested previously [31]. Because the relative spatial orientation of the two binding sites is unknown [32, 33], it is unclear whether Gαq and Gβγ are in contact with each other when bound to PLC-β3. When Gαq and Gβγ bind to the RGS domain of GRK2, the two subunits make no contact and lie essentially opposite sides of the central GRK2 molecule [34]. The absolute affinity of Gβγ for GTPγS-activated Gαq is low enough that it should not significantly sequester activated Gαq at the concentrations used here [28]. Does simultaneous binding of Gαq and Gβγ to PLC-β3 alter the conformation of either protein subunit? The ability of Gβγ to inhibit the Gαq GAP activity of PLC-β [28, 35] might involve such contact, but synergism between Gβγ and GTPγS-activated Gαq shows that synergism as such does not involve GAP inhibition.

General Mechanism for Synergistic Response by a Single Enzyme

The synergistic response of PLC-β3 can be described quantitatively by a simple two-state allosteric model that requires only that PLC-β3 exist in two interconvertible states with low and high intrinsic activities (Figure 2). It neither requires nor predicts any particular physical property of the two states or of the transition between them. Activation may reflect gross domain rearrangement, movement of an autoinhibitory structure, minor motion of an active site residue, or, as suggested for the PLCαs [32], reorientation with respect to the membrane bilayer. More broadly, a general two-state model can account for synergism regardless of whether regulation is allosteric or covalent. Noncovalent allosteric regulation of a protein that is also stimulated by phosphorylation, for example, can be described by the same conformational equilibria shown in Figure 2. Similarly, the model is applicable to signaling proteins that are not enzymes: transcription factors, channels, scaffolds, etc. Although any two-state model is a simplification of a protein’s dynamic structure, this model shows that synergism can be attained without supposing distinct conformations favored by each ligand or their combination.

The two-state model predicts synergism without demanding any direct interaction between the two ligands or any direct effect of one ligand upon the binding of the other. In terms of Figure 2, Gβγ does not change F and Gαq does not change G. Synergism occurs simply because the binding of both ligands favors the active state. There is no “higher-order coupling.” The two-state model was developed to deal with cooperative ligand binding [2, 3] and obviously predicts positive cooperativity of binding of the two ligands (Figure S1). Enhanced binding can result in physiologic synergism as one ligand allows another to act at a lower concentration than it
would otherwise (e.g., [4, 36, 37]). However, the synergism described here results from an increased population of the active state of the enzyme rather than just increased affinity for activating ligands.

Synergistic activation in a two-state system demands that the enzyme strongly favor the inactive state in the absence of ligand. J must be low, and this makes intuitive sense. Binding of each ligand drives the enzyme to its more active form with the free energy associated with its bias constant, F or G. This is true regardless of J. However, a low value of J provides a large enough dynamic range of activation that the addition of these free energies can be expressed as a synergistic response in net activity. Synergism therefore does not require any effect of one ligand upon the other ligand’s intrinsic bias for the active state. Each ligand contributes its own \( \Delta \Delta G \) to the conformational equilibrium, but synergistic activation does not require a “\( \Delta \Delta \Delta G \)” for ligand-ligand interaction. Such complex interactions surely occur for some enzymes, glycogen phosphorylase for example [38, 39], but they demand the explicit assumption of more and different stable conformational states, which in general is unnecessary.

Why is synergism observed so rarely if the simplest and most common model for allosteric predicts it? Again, the answer lies with the demand for a low value of J. Maximum synergism and J are approximately inversely proportional (Figure 5E). If an enzyme is even 1% active without ligands, its capacity for a synergistic response will be slight, and it will display no synergism at all unless the bias constants for the activators and their concentrations are fortuitously well matched. Most allosteric enzymes are stimulated less than 100-fold by their regulatory ligands, and far smaller stimulation can be important for cellular regulation. Yet, these proteins will not show detectable synergistic responses.

In contrast, decreasing intrinsic activation to 0.1% allows an enzyme to respond with robust synergism, as is the case for PLC-\( \beta \)-3. Maximum synergism will exceed 8-fold and will be observed for ligands that display a relatively broad range of bias constants. The concentration optima for synergism will depend on the bias constants, but high synergism will be observed over a > 10-fold range of activator concentrations and will be more than 2-fold for all relevant activator concentrations. This is the case for PLC-\( \beta \)-3 (Figure 4). Values of J < 0.001 further broaden the extent of synergism and the tolerance for divergent bias constants (Figure 5).

For the PLC-\( \beta \)-is, this analysis explains why PLC-\( \beta \)-3 responds synergistically to G\(_{q}\) and G\(_{\beta \gamma} \) but PLC-\( \beta \)-1 and PLC-\( \beta \)-2 do not. Although PLC-\( \beta \)-2 responds well to both G protein subunits, its intrinsic activity is too high, J = 0.15, and no combination of concentrations or bias constants will allow synergism. For PLC-\( \beta \)-1, synergism is limited because its intrinsic response to G\(_{\beta \gamma} \) is too low, even though it responds to G\(_{\beta \gamma} \) significantly both in cells and after purification.

Using the basal activation set point to determine whether an enzyme functions as a coincidence detector or merely as a dual responder offers distinct evolutionary advantages. Synergism can be acquired or lost by changing J only 10-fold, while retaining the same fractional (“\( \sim \) 10-fold”) responses to each regulatory input. An enzyme with J = 0.01 can respond to two ligands with almost a 100-fold dynamic range but display essentially no synergism. Alternatively, for J = 0.001, the protein will act as a sensitive coincidence detector in addition to providing a response to each ligand. An enzyme can evolve between these two regimes without sacrificing underlying allosteric regulation. Even absolute signaling activity can be retained with only minor changes in either catalytic activity (k\(_{cat}\)/K\(_{m}\) for the active state) or level of expression. In terms of cellular signaling, changing J in the range below 0.01 will have negligible practical effect on basal activity.

The general inverse dependence of synergism on an enzyme’s basal level of activity suggests that any enzyme that can be activated more than 500-fold (J < 0.002) is likely to display synergism among its activators. Examples include adenyl cyclases [40], some protein kinase C isoforms [41], and the Rac exchange factor P-Rex1 [42]. Novel synergisms should be detectable by identifying other highly regulated enzymes. Evaluating the behavior of these enzymes in cells should drive discovery of new synergisms, coincidence detectors, and biological AND gates.

Last, even though our data do not speak to the regulation of synergism by additional inputs, the allosteric model argues that synergism can be modulated best by controlling the value of J, perhaps with an added benefit of reducing basal activity. Modulation of J by other signaling mechanisms can thus convert an enzyme that responds independently to stimuli into a coincidence detector.

Experimental Procedures

Detailed experimental procedures are in the Supplemental Information.

All proteins were purified essentially as described [43]. G\(_{q}\) and G\(_{\beta \gamma} \) were finally concentrated by adsorption to Q-Sepharose and elution in 5 mg/ml 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) to minimize detergent in the PLC assay. G\(_{q}\) was activated with GTP-S [44], but incubation was extended to 5 hr such that G\(_{q}\) that did not bind GTP-S would be denatured and would not bind G\(_{\beta \gamma} \). G\(_{\beta \gamma} \) was used throughout except in Table SI, where other G\(_{\beta \gamma} \) isoforms were tested.

PLC activity was measured at 37°C by monitoring hydrolysis of \(^{3}H\)PIP\(_{2} \) on the surface of large unilamellar vesicles composed of PE:PS:PIP\(_{2} \) (20:4:1 molar ratio), roughly similar to the inner monolayer of the plasma membrane [43]. Activities are reported as moles of IP\(_{3} \) produced per min per mole of PLC. The concentration of free Ca\(^{2+} \) was adjusted with an EGTA buffer and the program Bound and Determined [45] and was 60 nM unless indicated otherwise. Because PLC-\( \beta \)-3 can be activated more than 10⁴-fold by combination of Ca\(^{2+} \), G\(_{\beta \gamma} \), and G\(_{q}\) (see Figure 1), assay time (2–40 min) and PLC-\( \beta \)-3 concentration (10–4000 pm) were adjusted for each assay to maintain linearity of activity with enzyme concentration, obtain accurately measurable PIP\(_{2} \) hydrolysis, prevent substrate depletion, and control free concentrations of G protein subunits. CHAPS inhibits stimulation of PLC-\( \beta \)-3 with IC\(_{50} \) = 100 \( \mu \)M. CHAPS was less than 20 \( \mu \)M in all assays and was equalized among all samples in each assay.

Supplemental Information

Supplemental Information includes five figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.06.013.

Acknowledgments

We thank multiple colleagues at University of Texas Southwestern for valuable discussion; William Seaman, Robert Rebres, and Tamara Roach (University of California San Francisco) for sharing unpublished results; Paul Sternweis (UT Southwestern) for the PLC-\( \beta \)-2 baculovirus and PLC-\( \beta \)-3 CDNAs; and Iain Frasier (National Institute of Allergy and Infectious Diseases) for the PLC-\( \beta \)-4 CDNAs. This work was supported by National Institutes of Health (NIH) grant R01GM030355 and an American Recovery and Reinvestment Act competing supplement, and by Welch Foundation grant I-0982.
References


Synergistic Activation of Phospholipase C-β3 by Gαq and Gβγ Describes a Simple Two-State Coincidence Detector

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Supplemental Inventory
5 supplemental figures with legends
3 supplemental tables
Supplemental Experimental Procedures
Supplemental References
Fig. S1
Figure S3
Figure S4
Figure S5
Table S1. Multiple Gβγ isoforms synergize with Gαq

<table>
<thead>
<tr>
<th>Gαq</th>
<th>Gβγ</th>
<th>PLC Activity (min⁻¹ ± SD)</th>
<th>Synergism Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>---</td>
<td>547 ± 4</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+Gβ1γ2</td>
<td>24 ± 3</td>
<td>3.9</td>
</tr>
<tr>
<td>+</td>
<td>+Gβ1γ2</td>
<td>2250 ± 52</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+Gβ1γ3</td>
<td>74 ± 1</td>
<td>4.6</td>
</tr>
<tr>
<td>+</td>
<td>+Gβ1γ3</td>
<td>2840 ± 222</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+Gβ1γ5</td>
<td>110 ± 9</td>
<td>4.5</td>
</tr>
<tr>
<td>+</td>
<td>+Gβ1γ5</td>
<td>2970 ± 189</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>+Gβ2γ5</td>
<td>66 ± 12</td>
<td>3.9</td>
</tr>
<tr>
<td>+</td>
<td>+Gβ2γ5</td>
<td>2370 ± 115</td>
<td></td>
</tr>
</tbody>
</table>

PLC-β3 was assayed in the presence of four different Gβγ isoforms, 5 nM each, with and without 0.5 nM Gαq. These Gβγ preparations were purified without the final anion exchange step and therefore produced lower activities because of excess detergent in the assays. Similar results, i.e., minimal difference in synergism ratios among the Gβγ preparations, were obtained in other experiments using other concentrations of Gαq and Gβγ.
Table S2. \(G_\alpha_q\) must be activated to synergize with \(G_\beta_\gamma\).

<table>
<thead>
<tr>
<th>Additions</th>
<th>PLC Activity (min(^{-1}))</th>
<th>Synergism ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>36 ± 3</td>
<td></td>
</tr>
<tr>
<td>20 nM (G_\alpha_q)-GDP</td>
<td>45 ± 3</td>
<td></td>
</tr>
<tr>
<td>20 nM (G_\beta_\gamma)</td>
<td>178 ± 29</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>242 ± 64</td>
<td>0.92</td>
</tr>
<tr>
<td>0.2 nM (G_\alpha_q)-GTP(\gamma)S</td>
<td>235 ± 8</td>
<td></td>
</tr>
<tr>
<td>20 nM (G_\beta_\gamma)</td>
<td>178 ± 29</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>1630 ± 60</td>
<td>5.8</td>
</tr>
<tr>
<td>20 nM (G_\alpha_q)-GDP</td>
<td>45 ± 3</td>
<td></td>
</tr>
<tr>
<td>30 nM (G_\beta_\gamma)</td>
<td>238 ± 28</td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>263 ± 16</td>
<td>0.93</td>
</tr>
</tbody>
</table>

PLC-\(\beta_3\) activity was measured in the presence of the G protein subunits shown. \(G_\alpha_q\) was bound either to GDP or GTP\(\gamma\)S prior to assay. Values are means, ± SD, of triplicate determinations except for \(G_\alpha_q\)-GDP, where \(n=6\). Activity for 20 nM \(G_\alpha_q\)-GDP alone is listed twice for clarity in the table. Data are representative of results of 3 separate experiments.
Table S3. Model parameters for PLC-β2 at 60 nM Ca^{2+}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>2500 ± 130 min⁻¹</td>
</tr>
<tr>
<td>J</td>
<td>0.15 ± 0.015</td>
</tr>
<tr>
<td>K_q</td>
<td>0.240 ± 0.062 nM⁻¹</td>
</tr>
<tr>
<td>F</td>
<td>8.2 ± 1.4</td>
</tr>
<tr>
<td>K_b</td>
<td>0.00061 ± 0.00037 nM⁻¹</td>
</tr>
<tr>
<td>G</td>
<td>410 ± 120</td>
</tr>
</tbody>
</table>

Values for the parameters, ± SE, of the allosteric model (Figure3) were estimated by fitting data from the experiment shown in Figure S2, but with additional data not shown included to improve the quality of the fit. Z is the maximum activity of the PLC-β2 under these assay conditions, with units of mol PIP2 hydrolyzed/min/(mol PLC).
Legends for supplemental figures

Figure S1. Go_q and Gβγ each decrease the EC_{50} value of the other for activation of PLC-β3. Data are from the experiment shown in Figure 3. The EC_{50} values are derived from mid-points (± 95% confidence limits) of individual concentration-activity curves for one G protein subunit at selected concentrations of the other. Fits were based on a single-site saturation equation. The solid lines are calculated using the parameters of Table 2, and show that each G protein subunit decreases the EC_{50} of the other approximately 19-fold. This relative change is equal to the calculated change in the equilibrium binding constant for one subunit that is caused by a saturating amount of the other.

Figure S2. PLC-β1, PLC-β2 and PLC-β4 do not display Go_q-Gβγ synergism. Activities of PLC-β1, PLC-β2 and PLC-β4 were assayed in the presence of Go_q, Gβγ or both, each at the concentrations shown below the graphs. Three sets of data, ± SD, are shown for each PLC-β isoform, and the fourth bar in each set shows the sum of activities measured with Go_q or Gβγ alone. PLC-β1 and PLC-β4 were assayed at 1 μM Ca^{2+} and PLC-β2 was assayed at 60 nM Ca^{2+}. These results are representative of multiple experiments at diverse concentrations of PLC-β, Ca^{2+} and each G protein subunit. Unstimulated activities in these experiments were PLC-β1, 72 min^{-1}; PLC-β2, 69 min^{-1}; PLC-β3, 1210 min^{-1}. For reference, unstimulated activity for PLC-β3 in the experiment of Figure 1B was 7 min^{-1} at 60 nM Ca^{2+} and 89 min^{-1} at 1 μM Ca^{2+}.

Figure S3. Independent (non-synergistic) regulation of PLC-β2 by Go_q and Gβγ. PLC-β2 activity was assayed at 60 nM Ca^{2+} over a range of concentrations of Go_q and Gβγ chosen to optimize fitting the data to the allosteric model of Figure 2. Activities are plotted against the concentration of Go_q (panel A) and Gβγ (panel B) at various fixed concentrations of the other subunit. The data sown in the graphs were selected from a set of 92 duplicate determinations. Solid lines are simulations based on the model and the parameter values obtained in the fit (Table S3).
Figure S4. Dependence of synergism on the concentrations of G protein subunits at varying values of the bias constants F and G. To determine the interplay of G protein concentration and the bias constants F and G, the dependence of synergism ratios on concentration was simulated for four F,G pairs. Values of $K_b$, $K_q$ and $J = 0.0015$ were from Table 2. Maximal synergism is constant for all conditions, but the steepness of the peak and its symmetry vary according to values of F and G. The upper left panel uses F,G values from the fitting to the data of Figure 3. The right two use either the higher or lower value for both F and G, and the lower left panel has F and G reversed from the experimentally determined values. For equal values of F and G (top panels), synergism peaks symmetrically at an intermediate concentration of each subunit. At high values of F and G, the peak is sharp. At lower values, the peak becomes a broad plateau, such that significant synergism is displayed over a wide range of activator concentrations. For unequal values of F and G (left panels), synergism remains high along a ridge that extends to very high concentrations of the G protein subunit with the lower bias constant. The ridge falls off at high concentrations of the subunit with the higher bias constant. The general dependence of peak symmetry on F and G is independent of J, but peaks become lower and more sharp as J increases (Figure 5).

Figure S5. Synergism is more pronounced and less sensitively dependent on F and G when G protein concentrations are low. The synergism ratio for PLC-$\beta$3 was simulated for varied concentrations of $G\alpha_q$ and $G\beta\gamma$ with $J=0.0015$ (Table 2). G protein concentrations are listed as reduced concentrations, the product of the actual concentration and the association equilibrium constant with reduced $[G\alpha_q]$ abbreviated as Q and reduced $[G\beta\gamma]$ abbreviated as B. The upper five panels, with the concentrations of $G\alpha_q$ and $G\beta\gamma$ equal, show how increasing the concentrations sharpens the peak and finally decreases the maximum synergism that can be attained. However, at very low G protein concentrations, higher values of F and or G are needed for synergism. Thus both axes are extended 8-fold in the upper left panel. When the concentration of one G protein subunit is, as shown in the lower left two panels, the peak becomes asymmetric. The effect is large; the G axis of the lower left panel is extended 5-fold.
Supplemental Experimental Procedures

Protein purification

N-terminally His<sub>6</sub>-tagged PLC-β1 (rat), PLC-β2 (mouse) and PLC-β3 (mouse) were purified as described for PLC-β1 [43]. PLC-β4 (human) was purified similarly but with slight modifications. PLC-β4 was extracted from Sf9 membranes with 1 M NaCl in buffer A (20mM NaHepes (pH 7.5), 1mM MgCl<sub>2</sub>, 5 mM 2-mercaptoethanol, 10 μg/mL leupeptin, 1 μg/ml aprotinin and 0.1 mM PMSF). Extracted protein was stirred with Ni-NTA resin (GE) for 2 h in the presence of 0.1% Lubrol and 5mM imidazole. The resin was washed sequentially with buffer A plus 1 M NaCl, 0.1 % Lubrol and 5 mM imidazole and with buffer A plus 100 mM NaCl and 5 mM imidazole until A<sub>280</sub> reached baseline. PLC was eluted with buffer A plus 100 mM NaCl and 150 mM imidazole. The eluate was diluted in buffer B (20 mM NaMES pH 6.0, 20 % glycerol, 1 mM DTT and 0.1mM EDTA) and loaded onto a Mono-S column that was equilibrated with buffer B. After washing the column with 100 mM NaCl in buffer, PLC was eluted by a gradient of 100-500 mM NaCl in Buffer B.

G<sub>α</sub>q was purified as described [43]. To decrease the detergent:protein ratio, purified G<sub>α</sub>q was diluted 5-fold in buffer C (20mM NaHepes (pH7.5), 0.1 mM EDTA, 1 mM DTT, 10 μM GDP, 0.5 % CHAPS and 1 mM MgCl<sub>2</sub>), adsorbed to Mono-Q and washed with buffer C to replace cholate with CHAPS. G<sub>α</sub>q was eluted with 300mM NaCl in buffer C, diluted 10-fold in buffer C, adsorbed to a 0.1 ml column of Q-Sepharose and eluted with 300mM NaCl in buffer C. For the experiment shown in Table S2, the G<sub>α</sub>q-GDP was this preparation with no additional treatment.

Gβγ was purified as described by Kozasa and Gilman [46], and then concentrated and switched to a CHAPS-containing buffer as was done for G<sub>α</sub>q. Gβ1γ2 was used for all experiments except those shown in Table S1.

Protein concentrations were determined by amido black binding [47].

G<sub>α</sub>q was activated before assay by incubation at 30 °C with 1mM GTPγS in 50 mM
NaHepes (pH 7.5), 100 mM NaCl, 4 mM MgCl₂, 1 mM DTT, 50 mM (NH₄)₂SO₄, 0.1 mg/ml BSA and 0.4 % CHAPS [44]. Incubation was extended to 5 h such that any Gα₉ that had not bound GTPγS would be denatured [44] and would be unable to chelate Gβγ in the PLC assay. Gα₉ was activated at the highest concentration possible to minimize detergent in the PLC assay. The fraction of Gα₉ bound to GTPγS was determined by monitoring bound [³⁵S]GTPγS.

**PLC assay, data analysis and fitting**

PLC activity was measured by monitoring hydrolysis of [³H]PIP₂ on the surface of large unilamellar vesicles (PE:PS:PIP₂, 20:4:1 molar ratio; 0.25 mM total phospholipid) [43,48]. Vesicles were prepared in 50 mM NaHepes (pH 7.5), 100 mM NaCl and 2 mM EGTA. PLC activity is proportional to the concentration of PIP₂ under these conditions. The assay times (2 - 40 min) and the concentration of PLC (10 - 4000 pM) were adjusted such that PIP₂ hydrolysis remained linear with time and PLC concentration. The concentration of PLC was also kept low enough that it did not substantially deplete the total concentration of activated Gα₉ in the assay. Assays were initiated by the addition of PLC at 37 °C. The final concentration of free Ca²⁺ in the assays was adjusted with an EGTA buffer that contained 2 mM EGTA and a concentration of CaCl₂ calculated according to the program Bound and Determined [45]. Data are expressed as moles of IP₃ produced per min per mole PLC.

The specific activities for PLC-βs under these assay conditions are relatively high, 45,000-60,000 min⁻¹ at optimal [Ca²⁺], [Gα₉] and [Gβγ] (Figure 1B). Because the PIP₂ substrate is 4 mol % of total lipids and activity increases linearly with substrate concentration in this range, higher activities would be observed with the higher PIP₂ concentrations that are frequently used. Cholate and CHAPS both inhibit PLC activity, with IC₅₀'s of 0.2 mM and 0.1 mM respectively under these conditions. The CHAPS concentration in the assays, derived from added G protein subunits, did not exceed 0.02 mM and was maintained equal among all samples within an assay.

Protein concentrations are reported in terms of the aqueous assay volume. However,
PLC-β, Gαq and Gβγ all bind to the surface of the substrate vesicles and occupy an annular shell volume about 5000-fold smaller. In addition, rotational mobility of the proteins is limited to one dimension. Values of K_b and K_q can be readjusted by factor to approximate actual protein-protein binding affinities, although the loss of two dimensions of rotational freedom mortgages thermodynamic comparisons with soluble reactions.

Assay data were fit using the Marquardt-Levenberg algorithm in either StatGraphic or SigmaPlot. Values for the parameters of the allosteric model (Figure 2) that are shown in Table 2 as “Matrix Fit” were derived by fitting a single data set for a PLC assay in which concentrations of Gαq and Gβγ were varied as shown in Figure 3. These data are representative of two other similar experiments. We were unable to fit data from all these experiments simultaneously because absolute activities vary among preparations of substrate vesicles. EC_{50} values for the G protein subunits vary 2- to 3-fold among experiments, presumably because of variable binding to the vesicles. Parameters for PLC-β2 shown in Table S3 were calculated similarly to those for PLC-β3.

Parameters for PLC-β3 shown as “4-point fits” (Table 2) were derived from three separate experiments in which PLC activity (v in the equations below) was assayed in the presence of saturating concentrations of Gαq, Gβγ (3 determinations in each experiment), both (6-8 determinations) or neither (4 determinations). Parameters were then calculated as Z = v for the combination of Gαq and Gβγ; J = v / Z without G protein; F = v / (J*(Z-v)) for Gαq alone; and G = v / (J*(Z-v)) for Gβγ alone. Data shown are the means of the values calculated from each experiment.
Supplemental References

