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Synergistic Activation of Phospholipase C-β3 by $G\alpha_q$ and Gβγ Describes a Simple Two-State Coincidence Detector

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Summary

Background—Receptors that couple to G_i and G_q often interact synergistically in cells to elicit cytosolic Ca^{2+} 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 G_i-G_q synergism derives from direct supra-additive stimulation of phospholipase C-β3 (PLC-β3) by G protein subunits Gβγ and $G\alpha_q$, the relevant components of the G_i and G_q 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

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Supplemental Information

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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 Ca^{2+} 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 Ca^{2+} 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 G_q and the other activates G_i, and synergism does not depend on which G_i- or G_q-coupled receptor initiates the signals. G_q and G_i both activate PLC-β isoforms, and the PLC reaction product, inositol-trisphosphate (IP_3), triggers Ca^{2+} release from the endoplasmic reticulum to the cytosol [21]. G_q stimulates PLC-β via its $G\alpha_q$ subunit, and G_i acts via its Gβγ subunit [21]. Several studies suggested that the mechanism of synergistic Ca^{2+} 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 Ca^{2+} signaling primarily requires the PLC-β3 isoform [10]. However, other work suggested that cellular G_i-G_q synergism involves interaction between the G proteins [25] or the IP_3 receptor [26], and its biochemical mechanism remained unknown.

We show here that purified PLC-β3 responds synergistically to stimulation by $G\alpha_q$ and Gβγ. Synergistic activation of PLC-β3 can exceed ten times the sum of the responses to the individual G protein subunits. Gβγ-$G\alpha_q$ synergism on PLC-β3 can thus quantitatively account for synergistic Ca^{2+} responses to G_i and G_q 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 $G\alpha_q$ 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 occur seven when both $G\alpha_q$ and Gβγ are tested at saturating concentrations.

The other PLC-β isoforms do not mediate synergistic Ca^{2+} responses in cells [10] or display synergism in vitro, even though they are structurally homologous to PLC-β3 and respond similarly to individual G proteins [21].

In general, why does one enzyme respond synergistically to two activators while another does not? We show by modeling and by analysis of PLC-β regulation that a superadditive

response by a single enzyme primarily depends on its having very low activity in the absence of stimulating ligand. Maximal attainable synergism by a simple two-state enzyme is approximately proportional to its intrinsic bias for the inactive state. A two-state enzyme whose intrinsic activation is ≥ 1% of maximal cannot display more than two-fold synergism, and it can do so only with ligands that are fortuitously matched in their efficacies and that are at near perfect concentrations. In contrast, an enzyme with intrinsic activity ≤ 0.1% will display synergism to most activators and will do so over a broad range of activator concentrations. Thus any allosteric enzyme with a large dynamic range of regulation will display a synergistic response to two or more activating ligands. Synergism, which is widely assumed to be a complex phenomenon requiring ligand-ligand interactions or multiple activity states, can be described by a simple two-state allosteric equilibrium.

Results

$G\alpha_q$ and Gβγ Stimulate PLC-β3 Superadditively

In many cells, simultaneous stimulation of receptors coupled to G_i and G_q produces a cytosolic Ca^{2+} transient that is much larger than the sum of the those elicited by the individual receptors. The Ca^{2+} signal presumably results from Ca^{2+} release from endoplasmic reticulum, which is triggered by IP_3 that is produced by the activity of PLC-β. To see whether the synergistic Ca^{2+} response in cells reflects direct synergistic activation of PLC-β3 by Gβγ and $G\alpha_q$, we measured the activity of purified PLC-β3 at increasing concentrations of GTPγS-activated $G\alpha_q$ and in the presence or absence of Gβ1γ2 (Figure 1A). Together, $G\alpha_q$ and Gβγ stimulated PLC-β3 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).

$$\text{Synergism} = \text{Act}_{a,b} / (\text{Act}_a + \text{Act}_b) \quad (1)$$

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 $G\alpha_q$ and Gβγ occurred over a wide range of $G\alpha_q$ concentrations, from 0.03 nM to 9 nM, which approaches saturation. The extent of direct $G\alpha_q$-Gβγ synergism on PLC-β3 can thus readily account for the 2- to 6-fold synergistic responses of cellular IP_3-Ca^{2+} pathways that have been described for simultaneous stimulation by G_q- and G_i-coupled receptors.

Superadditive stimulation of PLC-β3 by Gβγ and $G\alpha_q$ also resembles cellular G_i-G_q synergism qualitatively. Gβγ mediates PLC-β stimulation in cells, and cellular $G\alpha_i$-GDP sequesters Gβγ to terminate signaling after GTP hydrolysis [21,27]. Similarly, $G\alpha_i$-GDP blocked both stimulation of PLC-β3 by Gβγ and its potentiation of $G\alpha_q$ (Table 1). Multiple Gβγ dimers yield superadditive stimulation when added with $G\alpha_q$ (see Table S1, available online), consistent with the occurrence of synergistic responses in diverse cell types. Other experiments used only Gβ1γ2. Gβγ-$G\alpha_q$ synergism also requires activation of $G\alpha_q$ by GTP or a nonhydrolyzable analog (GTPγS); $G\alpha_q$-GDP neither stimulates PLC-β3 nor potentiates stimulation by Gβγ at the highest concentrations tested (Table S2). Hence, all other experiments shown here use $G\alpha_q$ that has been activated by GTPγS. Because $G\alpha_q$ activated by GTPγS or GTP binds Gβγ with relatively low affinity [28], Gβγ does not block its stimulation of PLC-βs.

$G\alpha_q$-$G\beta\gamma$ synergism was independent of Ca^{2+} concentration from well below that of resting cytosol (30 pM) to higher than usually reported for stimulated cells (10 μM) (Figure 1B). Responses to $G\alpha_q$ and $G\beta\gamma$ should therefore be potentiative continuously during a cytosolic Ca^{2+} transient. Ca^{2+} also had a negligible effect on the EC_{50} or Hill coefficient for either G protein subunit. Because PLC activity with either or both G protein subunits extrapolates to zero at low Ca^{2+}, Ca^{2+} appears not to alter the G protein-driven activation↔deactivation equilibrium but simply to act as an amplifier of PLC activity. We therefore used 60 nM Ca^{2+} for all PLC-β3 experiments as a reasonable value for resting cytosolic concentration.

$G\alpha_q$-$G\beta\gamma$ synergism is insensitive to multiple other variations in assay conditions (mole fraction of PIP_2, PLC concentration, temperature, detergent, ionic strength, and lipid surface composition; data not shown). G_q-G_i synergism can therefore reasonably be expected in any cell that expresses PLC-β3. Taken together, these data indicate that direct synergistic activation of PLC-β3 by $G\alpha_q$ and $G\beta\gamma$ can account for superadditive IP_3-Ca^{2+} signaling in cells; no other component or pathway is required.

General Allosteric Mechanism for Synergistic Enzyme Activation

Because activated $G\alpha_q$ and $G\beta\gamma$ both stimulate PLC-β3 noncovalently, we asked whether a simple two-state allosteric model for PLC-β3 activation can account for the markedly superadditive responses to these ligands. Such a mechanism, described in Figure 2, demands only that (1) PLC-β3 exists in two conformational states, active (P*) and inactive (P), in equilibrium described by the constant J; (2) that both $G\alpha_q$ and $G\beta\gamma$ bind reversibly and independently to PLC-β3 in either conformational state; and (3) that both G protein subunits bind more tightly to the P* conformation, as described by the bias constants F and G. This model is classically used to describe allosteric activation by individual ligands [2,3]. Note that this two-state allosteric mechanism is quite general: it neither requires nor suggests any particular property of the P* state that makes it more active than P, nor any biochemical mechanism for the P↔P* transition. Activation may represent substantial subunit rearrangement, minor movement of residues 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 $G\alpha_q$ and $G\beta\gamma$. The activity of PLC-β3 was measured over a wide range of concentrations of activated $G\alpha_q$ and $G\beta\gamma$, 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 $G\alpha_q$ and $G\beta\gamma$ (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 of $G\alpha_q$ and $G\beta\gamma$ for 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:

$$\text{Activity}=Z\bullet\frac{J+J\bullet F\bullet K_q\bullet\left[G\alpha_q\right]+J\bullet G\bullet K_b\bullet[G\beta\gamma]+J\bullet G\bullet K_b\bullet[G\beta\gamma]\bullet F\bullet K_q\bullet\left[G\alpha_q\right]}{J+J\bullet F\bullet K_q\bullet\left[G\alpha_q\right]+J\bullet G\bullet K_b\bullet[G\beta\gamma]+J\bullet G\bullet K_b\bullet[G\beta\gamma]\bullet F\bullet K_q\bullet\left[G\alpha_q\right]+1+K_q\bullet\left[G\alpha_q\right]+K_b\bullet[G\beta\gamma]+K_b\bullet[G\beta$$

(2)

The response of PLC-β3 to a matrix of concentrations of $G\alpha_q$ and Gβγ 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 $G\alpha_q$ and Gβγ 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 $G\alpha_q$, Gβγ, or both (Table 2). This method is independent of K_b, K_q, 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 $G\alpha_q$ 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 $G\alpha_q$ stimulates ~250-fold and saturating Gβγ stimulates about 50-fold. Combination of saturating $G\alpha_q$ and Gβγ together produced about 80% of theoretical total activation (~600-fold) (Table 2). Each subunit thus markedly potentiated PLC-β3 activation by the other. Gβγ and $G\alpha_q$ 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 Gβγ and remains above 2-fold at the highest concentrations of both subunits.

The extent and concentration dependence of $G\alpha_q$-Gβγ 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 Gβγ and $G\alpha_q$, with a peak value of 10. The ratio falls off at high $G\alpha_q$ concentrations but is significantly greater than 2.0 even at saturating concentrations of $G\alpha_q$ and Gβγ and remains above 1.0 at very low concentrations where activation is minimal. The Gβγ concentration did not have a marked effect on the maximally synergistic concentration of $G\alpha_q$, nor did $G\alpha_q$ alter the maximally synergistic concentration of Gβγ. 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.

Other PLC-β Isoforms Do Not Display $G\alpha_q$-Gβγ Synergism

Seaman and coworkers [10] reported that only the PLC-β3 isoform produces synergistic responses to G_i- and G_q-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 $G\alpha_q$ and $G\beta\gamma$. 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 $G\beta\gamma$ and $G\alpha_q$ 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 $G\alpha_q$ and $G\beta\gamma$, 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 ($J \geq 0.01$), 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 Figure S4). 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 $G\alpha_q$ 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 G_i and G_q. PLC-β2 responds well to both Gβγ and $G\alpha_q$. 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 5E). 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 Ca^{2+} concentrations, and it is known to be less sensitive to Gβγ than are the -β2 and -β3 isoforms [21]. For $G \leq 4$, simulations do not predict any synergism regardless of Gβγ and $G\alpha_q$ 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 $G\alpha_q$-Gβγ synergism. The unique ability of PLC-β3 to respond synergistically to $G\alpha_q$ 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.

G_i-G_q Synergism

This study shows that synergistic signaling by G_i- and G_q-coupled receptors can be explained by the superadditive response of PLC-β3 to stimulation by Gβγ and $G\alpha_q$. G_i-G_q 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, G_i provides the Gβγ because a relatively high Gβγ concentration is required (Figures 3 and 4) and only the G_i family heterotrimers are expressed at high enough levels and release their Gβγ adequately [21,30]. G_is 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 $G\alpha_q$ is quantitatively more than adequate to account for cellular G_i-G_q synergism over the range of cytosolic Ca^{2+} concentrations. Only PLC-β3 among the PLC-β isoforms displays this behavior, which agrees with the finding that only PLC-β3 permits G_i-G_q synergism in cells [10]. PLC-β3 is thus a sensitive cellular coincidence detector, one of few allosteric proteins that can act in this way. G_i-G_q synergism requires no other cellular proteins or pathways. By expression of this isoform, cells can switch between an additive response to G_i and G_q inputs and a coincidence detection mode.

Synergism demands that both $G\alpha_q$ and $G\beta\gamma$ bind simultaneously to nonoverlapping sites on PLC-β, as suggested previously [31]. Because the relative spatial orientation of the two binding sites is unknown [32,33], it is unclear whether $G\alpha_q$ and $G\beta\gamma$ are in contact with each other when bound to PLC-β3. When $G\alpha_q$ and $G\beta\gamma$ bind to the RGS domain of GRK2, the two subunits make no contact and lie on essentially opposite sides of the central GRK2 molecule [34]. The absolute affinity of $G\beta\gamma$ for GTPγS-activated $G\alpha_q$ is low enough that it should not significantly sequester activated $G\alpha_q$ at the concentrations used here [28]. Does simultaneous binding of $G\alpha_q$ and $G\beta\gamma$ to PLC-β3 alter the conformation of either G protein subunit? The ability of $G\beta\gamma$ to inhibit the G_q GAP activity of PLC-β [28,35] might involve such contact, but synergism between $G\beta\gamma$ and GTPγS-activated $G\alpha_q$ 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 PLCs [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, $G\beta\gamma$ does not change F and $G\alpha_q$ 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 ΔΔG to the conformational equilibrium, but synergistic activation does not require a "ΔΔΔG" 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 allostery 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-β3. 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-β3 (Figure 4). Values of $J < 0.001$ further broaden both the extent of synergism and the tolerance for divergent bias constants (Figure 5).

For the PLC-βs, this analysis explains why PLC-β3 responds synergistically to $G\alpha_q$ and $G\beta\gamma$ but PLC-β1 and PLC-β2 do not. Although PLC-β2 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-β1, 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 ("-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 adenylyl 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]. $G\alpha_q$ 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. $G\alpha_q$ was activated with GTPγS [44], but incubation was extended to 5 hr such that $G\alpha_q$ that did not bind GTPγS would be denatured and would not bind $G\beta\gamma$. $G\beta1\gamma2$ 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 $[^3H]PIP_2$ 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-β3 can be activated more than 10^4-fold by combination of Ca^{2+}, $G\beta\gamma$, and $G\alpha_q$ (see Figure 1), assay time (2–40 min) and PLC-β3 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-β3 with IC_{50} = 100 μM. CHAPS was less than 20 μM in all assays and was equalized among all samples in each assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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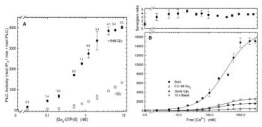

Figure 1.

Synergistic Activation of PLC-β3 by $G\alpha_q$ and Gβγ

(A) PLC-β3 activity was assayed at 60 nM [Ca^{2+}] with increasing concentrations of $G\alpha_q$ in the absence (open circles) or presence of 6 nM Gβγ (closed circles). The synergism ratio, the ratio of activities in the presence of both $G\alpha_q$ and Gβγ to the sum of the activities in the presence of each subunit alone, is given at each $G\alpha_q$ concentration.

(B) $G\alpha_q$-Gβγ synergism is independent of [Ca^{2+}]. Lower panel: PLC-β3 activity was assayed at various Ca^{2+} concentrations in the presence of 30 nM Gβγ (black triangles), 0.2 nM $G\alpha_q$ (white circles), or both 0.2 nM $G\alpha_q$ and 30 nM Gβγ (black circles). Basal activity in the absence of G protein subunits was also assayed, and is shown multiplied by 10 to distinguish it from baseline (open triangles). Zero Ca^{2+} represents 5 mM EGTA with no added Ca^{2+}. Upper panel: 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 activities in this experiment is greater than 2000-fold. The maximum activity shown for the combination of $G\alpha_q$ and Gβγ (both) is about one-third that in the presence of an optimal concentration of Gβγ. Error bars show standard deviation (SD).

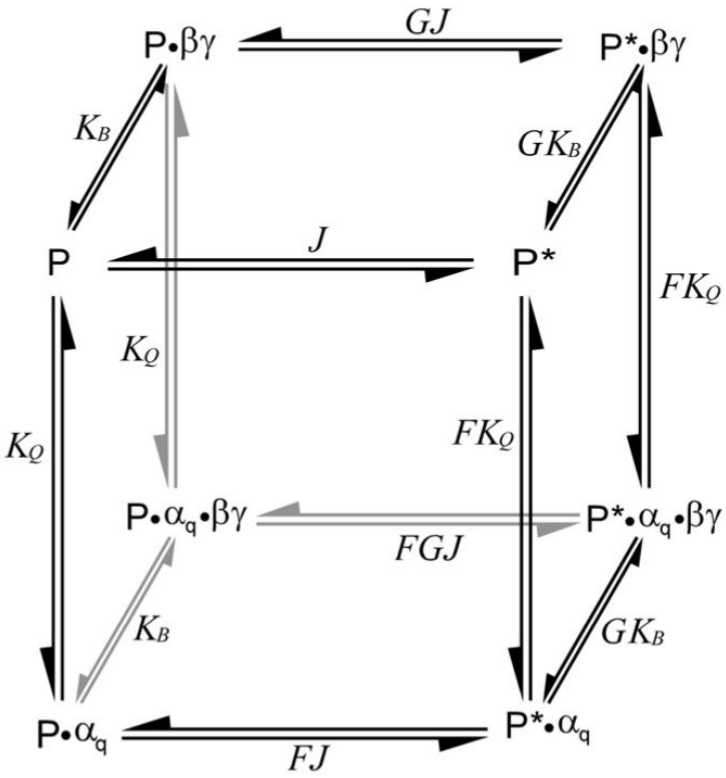

Figure 2.

A Two-State Allosteric Model for Synergistic Activation of PLC-β3

PLC can exist in one of two states, either relatively inactive (P) or highly active (P*), with the intrinsic conformational equilibrium described by the isomerization constant J. $G\alpha_q$ and $G\beta\gamma$ can bind to either state at nonoverlapping sites, with association constants K_q and K_b defined for the inactive (P) conformer. $G\alpha_q$ and $G\beta\gamma$, both allosteric activators, bind relatively more tightly to the active state, with their preference for P* over P described by the bias constants F and G.

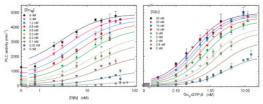

Figure 3.

Coordinate Regulation of PLC-β3 by $G\alpha_q$ and Gβγ

PLC-β3 activity was assayed at 60 nM Ca^{2+} over a range of concentrations of $G\alpha_q$ and Gβγ chosen to optimize fitting the data to the scheme shown in Figure 2. Activities are plotted against the concentration of $G\alpha_q$ (A) and Gβγ (B) at various fixed concentrations of the other subunit. One hundred data points, averages of duplicates, with ranges, are shown out of a total of 115. Solid lines are simulations based on the scheme in Figure 2 and the parameter values in Table 2.

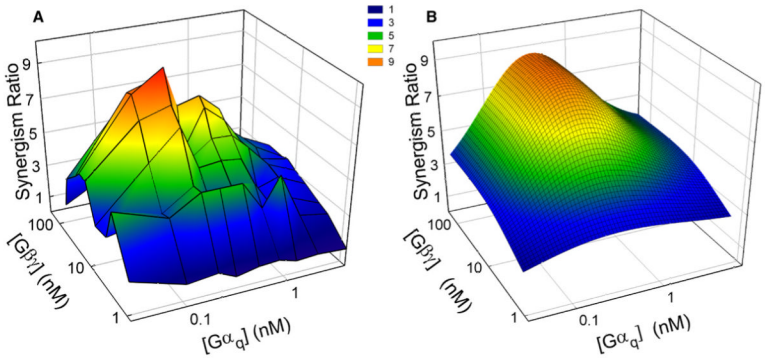

Figure 4.

$G\alpha_q$-$G\beta\gamma$ Synergism Is Maximal at Intermediate G Protein Concentrations

(A) The data from the experiment shown in Figure 2, PLC-β3 activities assayed over a range of concentrations of $G\alpha_q$ and $G\beta\gamma$, are replotted as synergism ratios, calculated as described in the legend to Figure 1. Each vertex on the surface represents a ratio calculated from the three assays (PLC-β3 plus $G\alpha_q$, $G\beta\gamma$, or both), each performed in duplicate. This plot is similar to those derived from two other similar experiments.

(B) Synergism ratios for the experiment in Figure 4A were simulated according to the allosteric model and the parameters shown in Table 2.

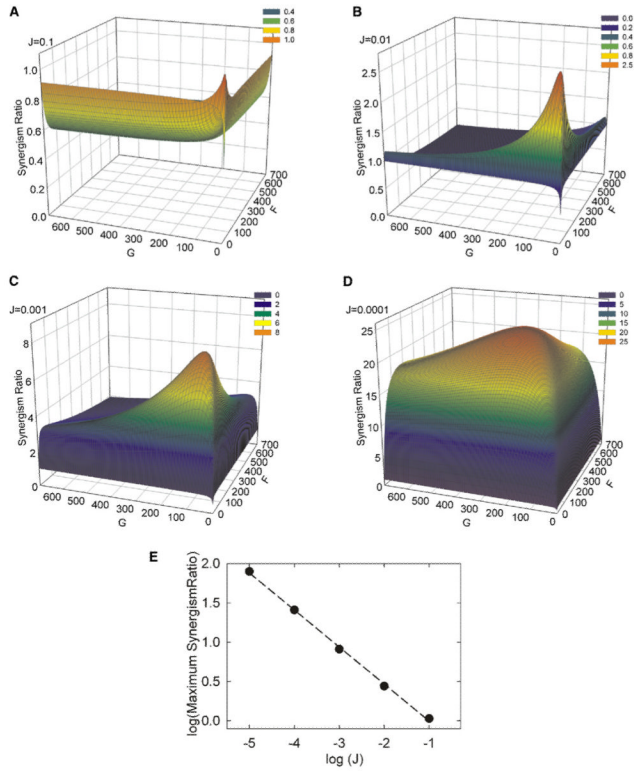

Figure 5.

Predicted Effect of the Spontaneous Activation Constant J on Synergistic Activation by two allosteric regulators

The activity of an enzyme that is activated by two ligands according to the two-state model (Figure 2) was simulated over a range of values of the bias constants F and G. For reference, J ~ 0.001 for PLC-β3 (Table 2). The graphs show calculated synergism ratios at four values of J: 0.1, 0.01, 0.001, and 0.0001. Note the scale differences among the synergism axes in each panel; the maximal synergism ratio for J = 0.1 is less than 1.0. The graph at the bottom shows the nearly inverse relationship between the maximal synergism ratio and J, with a straight line of best fit drawn for reference. For the simulations, the concentrations of the two activators were set equal to $1/K_q$ and $1/K_b$. Changing the concentrations alters the location of the maximum synergism ratio in the F-G plane but has no effect on its value over a wide range of concentrations (see Figures S4 and S5).

Table 1

$G\alpha_{i1}$-GDP Blocks $G\beta\gamma$-$G\alpha_q$ Synergism

[$G\alpha_q$-GTPγS] (nM)	[Gβγ] (nM)	[$G\alpha_{i1}$-GDP] (nM)	Synergism Ratio
0.2	10	0	4.6
0.2	10	30	0.59
0.2	10	30; heated	4.6
0.2	5	0	4.7
0.2	5	15	0.91
0.2	5	15; heated	4.4

Synergism ratios were determined at 0.2 nM GTPγS-activated $G\alpha_q$ and two concentrations of Gβγ, with or without a 3-fold molar excess of GDP-bound $G\alpha_{i1}$. Controls contained $G\alpha_{i1}$ 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 $G\alpha_i$ control. $G\alpha_{i1}$-GDP also blocked stimulation by Gβγ alone (not shown).

Table 2

Allosteric Model Parameters for PLC-b3

	Matrix Fit	4-Point Fit
Z	5300 ± 130 min^{-1}	
J	0.00150 ± 0.00047	0.00094 ± 0.00002
K_q	0.220 ± 0.042 nM^{-1}	
F	434 ± 154	1700 ± 300
K_b	0.0307 ± 0.0056 nM^{-1}	
G	45.9 ± 9.0	41 ± 1.1

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 Ca^{2+}. 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 phos-pholipid substrate vesicles. 4-point fit parameters (average of three experiments, ± SD) were calculated from activities obtained at saturating values of $G\alpha_q$, $G\beta\gamma$, both, or neither. The 4-point fit is independent of Z, K_q, and K_b. Details are in the Supplemental Information.