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Article

Cooperative Regulation of PIP5K by β -Arrestin–GPCR Complexes at Clathrin-Coated Pits Demonstrated by Mathematical Modeling

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Abstract

Background/Objectives: Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂ or PIP₂) is a low-abundance plasma membrane phosphoinositide, an essential factor for clathrin-mediated endocytosis. Activation of G_q-protein-coupled receptors (G_qPCR) such as protease-activated receptor 2 (PAR2) triggers phospholipase C-mediated hydrolysis of PIP₂, creating a paradox in which the lipid is simultaneously consumed for signaling yet required for receptor internalization. I previously showed that β -arrestin promotes PIP₂ regeneration by recruiting Phosphatidylinositol-4-phosphate 5-kinase (PIP5K), partially resolving this paradox. However, the modest hyperactivation of PIP5K (~2–3 fold) and the hump-like kinetics of PIP₂ recovery following β -arrestin knockdown indicated that additional regulatory mechanisms were involved. **Methods:** Here, my revised model incorporates contributions from both preexisting and newly forming clathrin-coated pits (CCPs) using Virtual Cell. **Results:** I find that CCPs provide a spatially confined environment that promotes clustering of receptor– β -arrestin complexes, thereby amplifying cooperatively PIP5K activity through weak interaction between PIP5K and β -arrestins in the receptor– β -arrestin-CCP complexes. This cooperative mechanism accounts for nonlinear PIP₂ recovery kinetics, delayed kinase activation under β -arrestin knockdown conditions, and the sufficiency of modest PIP5K activation to restore plasma membrane lipid pools. These findings suggest that transient and spatially restricted protein interactions, often considered weak or biologically insignificant, play decisive roles in lipid metabolism and receptor trafficking. **Conclusions:** This framework provides a mechanistic resolution to the paradox of simultaneous PIP₂ hydrolysis and receptor internalization and highlights the importance of cooperative dynamics in maintaining plasma membrane signaling competence.

Keywords: cooperative kinase activation; PIP5K; β -arrestin; endocytosis; G_qPCR

1. Introduction

Phosphoinositides are critical regulators of signaling, trafficking, and cytoskeletal dynamics although they are minor constituents of cellular membranes [1]. Among them, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂ or PIP₂) plays a central role in clathrin-mediated endocytosis by recruiting adaptor proteins, clathrin, and actin regulators [2–5]. Acute perturbation of PIP₂ levels demonstrates its indispensability: optogenetic or enzymatic depletion of PIP₂ blocks clathrin-coated pit (CCP) initiation and receptor internalization [6–8]. These studies establish PIP₂ not only as a structural lipid but also as a dynamic regulator of receptor trafficking.

G-protein-coupled receptors (GPCRs) comprise the largest family of cell-surface receptors and mediate responses to diverse extracellular cues. Upon activation, G_q-coupled GPCRs stimulate phospholipase C β (PLC β), which hydrolyzes PIP₂ into diacylglycerol (DAG) and inositol trisphosphate (IP₃), thereby reducing plasma membrane PIP₂ levels [9,10]. Yet GPCRs such as protease-activated receptor 2 (PAR2) undergo clathrin-dependent endocytosis for desensitization and trafficking, a process that requires PIP₂. This apparent contradiction — that G_q signaling

consumes PIP₂ while GPCR endocytosis depends on it — represents a long-standing paradox in GPCR biology [11,12].

CCPs are increasingly recognized as more than passive vesicle carriers. CCPs serve as multifunctional signaling microdomains that integrate receptor trafficking with downstream signal transduction [13,14]. For example, they provide a spatial platform for MAPK activation downstream of GPCRs [15]. β -arrestins are key to this dual role. They not only terminate G-protein signaling but also scaffold MAPK components and link receptors to clathrin and AP-2 [16]. Live-cell and single-molecule imaging studies have shown that β -arrestin–receptor complexes display dynamic lifetimes and can persist within CCPs, where they regulate both receptor endocytosis and signal propagation [17–19].

The previous work provided evidence that β -arrestins contribute directly to the regeneration of PIP₂ at the plasma membrane following PAR2 activation [20]. Using lipid biosensors, I found that β -arrestin knockdown impaired PIP₂ recovery. Further experimental results suggested that phosphatidylinositol 4-phosphate 5-kinase (PIP5K), the kinase that synthesizes PIP₂ from PI(4)P, is recruited to β -arrestin–receptor–clathrin complexes, linking receptor signaling to localized lipid metabolism [21]. These findings offered a potential solution to the PIP₂ paradox. However, several observations remain unexplained: the degree of PIP5K hyperactivation was modest (2–3 fold), and β -arrestin knockdown produced a hump-like trajectory in PIP₂ recovery inconsistent with linear recruitment models.

In the present study, I tested the hypothesis that PIP5K activity is cooperatively modulated within CCPs using mathematical modeling. I proposed that CCPs can provide spatial confinement and clustering of receptor– β -arrestin complexes that amplify cooperatively the PIP5K activity. Through mathematical modeling, I showed that transient, weak protein–protein interactions at CCPs can be sufficient to reproduce the nonlinear kinetics of PIP₂ recovery, the hump phenotype of β -arrestin knockdown, and the modest hyperactivation of PIP5K. These results advance a conceptual framework in which both stable and transient protein interactions at CCPs govern lipid metabolism, thereby ensuring that GPCR signaling and endocytosis remain tightly coupled and reversible.

2. Materials and Methods

2.1. Model Overview

A mathematical model was developed to describe cooperative regulation of PIP5K activity during recovery of PIP₂ following activation of G_q-coupled PAR2. The model builds upon our previous framework [20] in which PIP₂ hydrolysis and resynthesis kinetics were represented as coupled differential equations describing the balance between phospholipase C (PLC)–mediated degradation and PIP5K-dependent synthesis. The current model introduces additional parameters to account for cooperative enzyme activation and the distinct contributions of newly forming versus preexisting CCPs prior to endocytosis of receptor by dynamin action.

Model Assumptions: 1) Activation of PAR2 by 100 μ M activating peptide (AP) induces rapid PIP₂ hydrolysis by PLC, leading to transient depletion of the lipid pool at the plasma membrane. 2) PIP5K activity recovers PIP₂ levels with two distinct components: one associated with newly forming CCPs and another with preexisting CCPs. 3) β -arrestin–dependent recruitment of PIP5K to CCPs modulates enzyme activity cooperatively, described by Hill-type equations with coefficients n and m , representing newly forming and preexisting CCPs, respectively. 4) The kinetic equations assume well-mixed conditions and constant total enzyme concentration. Spatial heterogeneity and stochastic fluctuations were not explicitly modeled but are implicitly reflected in the cooperative parameters.

2.2. Mathematical Formulation

The cooperative activation term was expressed as:

$$\text{Stim_5K_max}(n, m, a, b) = \text{basal} + a \cdot \frac{[\text{RLPAAPC}]^n}{[\text{RLPAAPC}]^n + K_a^n} + b \cdot \frac{[\text{RLPA_CCP}]^m}{[\text{RLPA_CCP}]^m + K_b^m},$$

where, *RLPAAPC* represents the ligand (L)–bound, phosphorylated (P) GPCR (R) complexed with β -arrestin (A) that nucleates newly forming CCPs through adaptor (AP)–clathrin (C) interactions. *RLPA_CCP* represents the ligand (L)-bound, phosphorylated (P) GPCR (R) complexed with β -arrestin (A) associated with a preexisting CCP. *a* and *b* are weighting constants describing the fractional contributions of newly forming and preexisting CCPs, respectively. *K_a* and *K_b* are apparent dissociation constants for each process. *n* and *m* are Hill coefficients indicating the degree of cooperativity for each component.

2.3. Parameter Selection

Parameter values were based on previously reported measurements and adjusted to match experimentally observed kinetics of PIP₂ depletion and recovery after PAR2 activation [20,21]. The Hill coefficients (*n*, *m*) were varied between 1 and 6 to explore the effects of cooperativity. Weighting constants (*a*, *b*) were set to evaluate the relative contributions of newly forming and preexisting CCPs. All parameters and equations for reactions were described in Tables 1 and 2.

Simulation and Analysis: All simulations were performed in Virtual Cell. Plots were generated using the Origin pro (OriginLab Corp. MA, USA) for figures.

β -Arrestin Knockdown Simulations: To simulate β -arrestin knockdown, the amount of cytosolic β -arrestin was reduced by 20%, corresponding to the experimentally observed decrease in PIP₂ recovery following β -arrestin depletion. This reduction delayed PIP₂ recovery and produced the characteristic “hump” phenotype in the simulated time course.

Table 1. Model initial conditions and parameters.

Species/constants	Value	Rationale
R for PAR2	5,000 μm^{-2}	Receptor density [20]. If the receptors were able to dissolve in cytosol, it would be 5 μM .
G (G-protein)	40 μm^{-2}	Number of free G-protein at the plasma membrane
PLC	10 μm^{-2}	Number of free PLC at the plasma membrane
PI	140,000 μm^{-2}	Number of free PI at the plasma membrane
PIP	3,600 μm^{-2}	Number free PIP at the plasma membrane
PIP ₂	5,000 μm^{-2}	Number of free PIP ₂ at the plasma membrane
IP ₃	0.015 μM	IP ₃ at cytosol before receptor activation: Steady state balance from PLC and IP ₃ ase
DAG at PM	23 μm^{-2}	Number of DAG at the plasma membrane before receptor activation
β -arrestin (cytosol) = β -arrestin1+ β -arrestin2	15 μM	β -arrestin 1 (Arrestin 1) and β -arrestin 2 (Arrestin 2) have 7.5 μM , respectively, to leave proper amount β -arrestins in the cytosol after their binding to the PAR2 at the plasma membrane.

PKC_cyto (PKC Cytosol)	1 μM	The concentration was chosen to make reasonable fitting of DAG bound PKC
GRK	600	Fixed value to similar to the peak value of PKC_DAG
Weighting_factor_PKC_DAG or Weighting_factor_GRK	0.5	Active PKC (PKC_DAG) gives the same contribution to phosphorylation of ligand bound receptor as GRK.
$k_{\text{f_PKC_DAG}}$	$0.02 \mu\text{M}^{-1}\text{s}^{-1}$	Forward rate constant for binding of PKC and DAG
$K_{\text{r_PKC_DAG}}$	0.06 s^{-1}	Reverse rate constant for dissociation of DAG from PKC, giving $K_{\text{d}} = 3 \mu\text{M}$
Ca^{2+} (cytosol)	$0.13 \mu\text{M}$	Typical intracellular Ca^{2+} level is $0.1\text{-}0.2 \mu\text{M}$
foldPIP ₂	3	Making the size of the total PIP ₂ (bound plus free) 3 times the free pool
k_{4K} (basal)	0.00078 s^{-1}	$\text{FoldPIP}_2 \cdot 0.00026 \text{ s}^{-1}$
k_{5K} (basal)	0.06 s^{-1}	$\text{FoldPIP}_2 \cdot 0.02 \text{ s}^{-1}$
k_{4K} (stimulated)	$6 \cdot k_{\text{4K}}$ (basal)	To fit the recovery kinetics of PIP ₂ probe
k_{5K} (stimulated)	$3 \cdot k_{\text{5K}}$ (basal)	To fit the recovery kinetics of PIP ₂ probe
k_{4P} (basal)	0.03 s^{-1}	$0.03 \text{ s}^{-1} \cdot k_{\text{4r_basal}}$ ($k_{\text{4r_basal}} = 1$)
k_{5P} (basal)	0.014 s^{-1}	$0.014 \text{ s}^{-1} \cdot k_{\text{5r_basal}}$ ($k_{\text{5r_basal}} = 1$)
k_{4P} (stimulated)	0.19 s^{-1}	$0.03 \text{ s}^{-1} \cdot k_{\text{4r_stim}}$ ($k_{\text{4r_stim}} = 6.25$)
k_{5P} (stimulated)	0.042 s^{-1}	$0.014 \text{ s}^{-1} \cdot k_{\text{5r_stim}}$ ($k_{\text{5r_stim}} = 3$)
$k_{\text{f_RL}}$	$0.75 \mu\text{m}^2 \cdot \text{molecules}^{-1} \cdot \text{s}^{-1}$	Rate constant for phosphorylation of ligand-bound receptor
$k_{\text{r_RLP}}$	0.0125 s^{-1}	Rate constant for dephosphorylation of ligand-bound phosphorylated receptor
$k_{\text{f_R}}$ (basal)	0.0001 s^{-1}	Basal phosphorylation rate constant of receptor
$k_{\text{r_RP}}$ (basal)	2 s^{-1}	Basal dephosphorylation rate constant of phosphorylated receptor
$k_{\text{f_R}}$ (stimulated)	10 s^{-1}	Stimulated phosphorylation rate constant of receptor
$k_{\text{r_RP}}$ (stimulated)	4 s^{-1}	Stimulated dephosphorylation rate constant of receptor. We assumed that rate of dephosphorylation of RP doubles after ligand treatment
$k_{\text{f_L2}}$	$0.09333 \mu\text{M}^{-1} \cdot \text{s}^{-1}$	Binding rate constant of ligand to RP, depending on the dissociation constant (K_{L2}) and dissociation rate constant ($k_{\text{r_L2}}$)

k_{r_L2}	5.6 s^{-1}	Dissociation rate constant of ligand from RP, assuming that it has same dissociation rate constant compared to the native receptor (R)
K_{L2}	$60\text{ }\mu\text{M}$	Dissociation constant of ligand bound to RP, which has slightly lower affinity compared to native receptor (R) based on the supplementary data
k_{f_RLP}	0.003 s^{-1}	Rate constant of β -arrestin 2 binding to phosphorylated ligand-bound receptor to make best fitting compared to Figures 4F and 8F. For β -arrestin 1, we used 0.006 s^{-1} .
$k_{r_RLPA1}\text{ or }k_{r_RLPA2}$	10^{-6} s^{-1}	Dissociation rate constant of β -arrestin from phosphorylated ligand-bound receptor. Same for β -arrestin 1 and 2
$K_{d_arrestin\ 1_RLP_PIP2}$ $K_{d_arrestin\ 2_RLP_PIP2}$ or	$5000\text{ molecules}\cdot\mu\text{m}^{-2}$	Dissociation constants of β -arrestin 1 or β -arrestin 2 from RLPA1 or RLPA1 complexes, respectively
K_{d_PIP2}	$5000\text{ molecules}\cdot\mu\text{m}^{-2}$	Dissociation constant of PIP2 dependent clathrin/AP2 binding to the RLPA1 or RLPA2 complexes
$k_{f_RLPA1}\text{ or }k_{f_RLPA2}$	$0.001\text{ }\mu\text{m}^{-2}\cdot\text{s}^{-1}$	Binding rate constants of RLPA1 or RLPA2 to clathrin/AP2 bound RLPA complexes (RLPAAPC)
$k_{r_RLPAAPC}$	0.00001 s^{-1}	Rate constant of clathrin/AP2 dissociation from PLPAAPC complex
k_{f_CCP}	$0.01\text{ molecules}\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}\cdot\mu\text{M}^{-1}$	Binding rate constant of RLPA1 or RLPA2 to preexisting CCP
k_{r_CCP}	0.00006 s^{-1}	Dissociation rate constant of RLPA1 or RLPA2 from preexisting CCP
$k_{f_ClathrinAP2}$	$0.01\text{ molecules}\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}\cdot\mu\text{M}^{-1}$	Binding rate constant of clathrin/AP2 to form preexisting CCP
$k_{r_ClathrinAP2}$	0.00006 s^{-1}	Dissociation constant of clathrin/AP2 from preexisting CCP
$k_{endocytosis}$	$0.001\text{ }\mu\text{M}$	Binding rate constant of dynamin for receptor endocytosis
k_{fail}	$0.01\text{ molecules}\cdot\mu\text{m}^{-2}\cdot\text{s}^{-1}$	Dissociation constant of dynamin for failed endocytosis

$$K_d_{dynamain} = \frac{[PIP2]^4}{([PIP2]^4 + K_d_{PIP2}^4)}$$

Where, $K_d_{PIP2} = 2500$ molecules/ μm^2 . Dissociation constant of dynamin molecules from endocytic vesicles which have cooperative enhancing mechanisms by PIP2.

The rate constant for the PI4K (k_{4K} during stimulation) = foldPIP2*($k_{4K_rest} + (stim_{4K} - k_{4K_rest} + k_{4K_basal}) * scale_{4K} * (1 - exp(-t/tau_{on}))$). Where, tau_on = 1 s, foldPIP2 = 3, scale_4K = 0.75, $k_{4K_rest} = 0.00026 s^{-1}$, $k_{4K_basal} = 0.0002353 s^{-1}$, $stim_{4K} = 0.00117 s^{-1}$.
The rate constant for the PIP5K (k_{5K} during stimulation) = foldPIP2*($k_{5K_rest} + (stim_{5K} - k_{5K_rest} + k_{5K_basal}) * scale_{5K} * (1 - exp(-t/tau_{on}))$), where tau_on = 1 s, foldPIP2 = 3, scale_5K = 1, $k_{5K_rest} = 0.02 s^{-1}$, $k_{5K_basal} = 0.0181 s^{-1}$, $stim_{5K} = \frac{Stim_{5K_max}}{(1+exp^{4.86})}$.

Where, Stim_5K_max =
 $stim_{5K_max_basal} + stim_{5K_max_constant_a} * beta_arrestin_contribution_a * \frac{[RLPAAPC]^n}{([RLPAAPC]^n + K_d_arrestin_PIP5K^n)}$
+ $stim_{5K_max_constant_b} * beta_arrestin_contribution_b * \frac{[RLPA_CCP]^m}{([RLPA_CCP]^m + K_d_RLPA_CCP^m)}$
Where, stim_5K_basal = 3, stim_5K_max_constant_a = 3, beta_arrestin_contribution_a = 0.8, Stim_5K_max_constant_b = 6, beta_arrestin_contribution_b = 0.2.

Table 2. Model differential equations.

Reaction	Flux
PI to PI(4)P	$k_{4K}*[PI] - k_{4P}*[PIP]$
PI(4)P to PI(4,5)P2	$k_{5K}*[PIP] - k_{5P}*[PIP2]$
PLC (basal)	$[PIP2]*(PLC_basal + k_{PLC}*foldPIP2*Ga_GTP_PLC)$, where PLC_basal = 0.0001 s ⁻¹ , $k_{PLC} = 0.2 \mu m^2 molecule^{-1} s^{-1}$, and foldPIP2 = 3
PLC (stimulated)	$[PIP2]*(PLC_basal + k_{PLC}*foldPIP2*Ga_GTP_PLC) + [PIP2]*PLC_stim*Ga_GTP_PLC*[Ca_C/(Ca_C+K_d_PLC_Ca)]$, where PLC_basal = 0.0001 s ⁻¹ , $k_{PLC} = 0.2 \mu m^2 molecule^{-1} s^{-1}$, foldPIP2 = 3, where PLC_stim = 7 s ⁻¹ , $K_d_PLC_Ca = 1 \mu M$
RL to RLP	$k_{f_RL}*[RL]*(weighting_factor_PKC_DAG*[PKC_DAG] + weighting_factor_GRK*[GRK]) - k_{r_RLP}*[RLP]$
PKC to active PKC	$k_{f_PKC_DAG}*[PKC]*[DAG] - k_{r_PKC_DAG}*[PKC_DAG]$
R to RP	$k_{f_R}*[R]*[PKC_DAG] - k_{r_RP}*[RP]$
RP to RLP	$k_{f_L2}*AP_Ex*[RP] - k_{r_L2}*[RLP]$
RLP to RLPA1	$\frac{k_{f_RLP}*[RLP]*[Arrestin1]*[PIP2]}{([PIP2] + K_d_arrestin1_RLP_PIP2)} - k_{r_RLPA1}*[RLPA1]$
RLP to RLPA2	$\frac{k_{f_RLP}*[RLP]*[Arrestin2]*[PIP2]}{([PIP2] + K_d_arrestin2_RLP_PIP2)} - k_{r_RLPA2}*[RLPA2]$
RLPA1 to RLPAAPC	$\frac{k_{f_RLPA1}*[RLPA1]*[ClathrinAP2]*[PIP2]^3}{([PIP2]^3 + (K_d_PIP2)^3)} - k_{r_RLPAAPC}*[RLPAAPC]$
RLPA2 to RLPAAPC	$\frac{k_{f_RLPA2}*[RLPA2]*[ClathrinAP2]*[PIP2]^3}{([PIP2]^3 + (K_d_PIP2)^3)} - k_{r_RLPAAPC}*[RLPAAPC]$
RLPA1 & RLPA2 to RLPA_CCP	$k_{f_CCP}*[CCP]*[RLPA1]*[RLPA2] - k_{r_CCP}*[RLPA_CCP]$
ClathrinAP2 to CCP	$k_{f_ClathrinAP2}*[ClathrinAP2] - k_{r_ClathrinAP2}*[CCP]$
RLPAAPC to vesicle	$\frac{k_{endocytosis}*[ClathrinAP2]*[dynamain]}{([dynamain] + K_d_dynamain)} - k_{r_fail}*[vesicle]$

Many additional reactions which have been already described in our previous paper are not on this table. The parameters are in previous papers [20]. I added arrestin/clathrin/AP2/dynamin dependent endocytosis mechanism in the current model.

3. Results

Using a simple diagram, I summarized previous findings [21] as shown in Figure 1A. According to a new simulation result, one modification was localization of PIP5K among the β -arrestin-GPCR complexes at the CCP to describe two major findings: 1) modest hyperactivation of PIP5K after desensitization of PAR2 and 2) hump formation of PIP₂ level while β -arrestin was knocked down using siRNA.

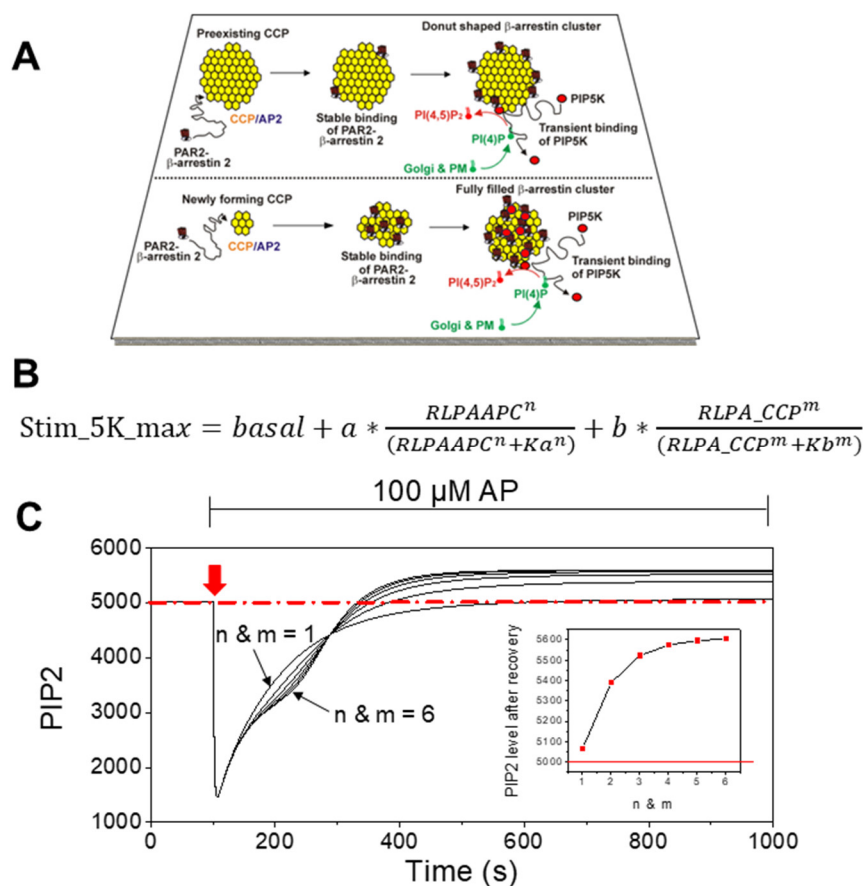


Figure 1. Cooperative regulation of PIP5K activity by β -arrestin-GPCR-AP2-clathrin-coated pit complexes. (A) Schematic representation of β -arrestin-GPCR complexes binding to clathrin-coated pits (CCPs). This diagram was modified from previous publication by the author [21]. Top: binding to a preexisting CCP. Bottom: recruitment into a newly forming CCP initiated by adaptor protein 2 (AP2)-clathrin assembly. (B) Parameters used to describe cooperative effects of β -arrestin-GPCR complexes within CCPs on PIP5K activity. *RLPAAPC* denotes the state in which a ligand-bound (RL), phosphorylated (P) GPCR complexed with β -arrestin (A) nucleates a newly forming CCP through AP2-clathrin (APC) assembly. *RLPA_CCP* represents the state in which the same receptor complex associates with a preexisting CCP. Cooperative constants n and m describe the degree of cooperative activation in each state (*RLPAAPC* and *RLPA_CCP*, respectively). The weighting a and b constants indicate relative contribution of preexisting and newly forming CCP, respectively. The K_a and K_b are apparent dissociation constants for cooperative reactions. (C) Simulated hydrolysis of PIP₂ in response to PAR2 activation by 100 μ M activating peptide (AP), as a function of the cooperative constants (n and m). Each curve represents PIP₂ dynamics at values of n and m ranging from 1 (no cooperativity) to 6 (high cooperativity).

To build upon our previous findings, we first examined the dynamics of PIP₂ following PAR2 activation. Using a PIP₂-sensitive PH probe, we observed that basal PIP₂ levels after desensitization of PAR2 exhibited an overshoot [21]. Recovery of PIP₂ was markedly impaired when β -arrestin expression was reduced by siRNA, producing a pronounced hump-like trajectory. This data suggested that an additional regulatory mechanism must be incorporated.

Interestingly, PIP5K activity was enhanced only 2–3 fold following PAR2 activation, a degree of hyperactivation that is difficult to reconcile with a simple conformational change mechanism which makes order of magnitude different activity of enzymes. This observation led us to hypothesize that the spatial organization of CCPs could play a critical role in modulating lipid kinase activity. Consistent with this idea, we found that PAR2 associated with both preexisting and newly forming CCPs. However, PAR2 was confined to the periphery of preexisting CCPs, whereas newly forming CCPs dynamically recruited PAR2- β -arrestin complexes together with endocytic machinery. These findings pointed out to de novo CCP formation as a major driver of PIP5K activation.

To capture these features, we developed a revised mathematical model (Figure 1B).

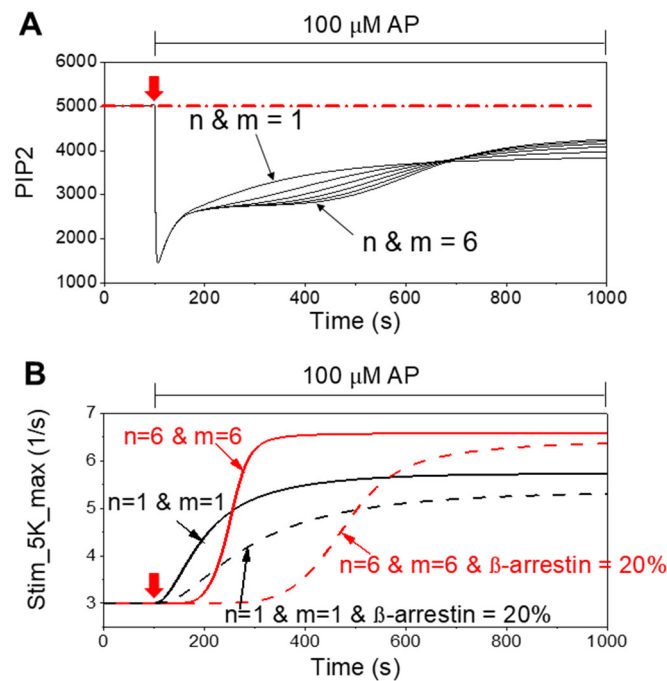


Figure 2. Cooperative PIP5K activity accounts for hump formation in PIP₂ recovery following β -arrestin knockdown. (A) Simulation of PIP₂ recovery after PAR2 desensitization at varying cooperative constants. When the cooperative constants (n and m) were set to 6, the hump in PIP₂ recovery was most pronounced. (B) Effect of β -arrestin knockdown on PIP5K activity. *Stim_5K_max* represents maximal PIP5K activity. Following PAR2 activation, β -arrestin knockdown by 20% caused a delayed rise in PIP5K activity and PIP₂ recovery. In both control and β -arrestin knockdown conditions, kinase activity was elevated by $\sim 30\%$ more compared with the n and $m = 1$ case, owing to the coherent increase contributed by the two terms representing preexisting and newly forming CCPs.

Unlike my previous framework, which treated PIP5K stimulation as a constant, the updated model incorporated dynamic terms reflecting CCP formation. The second and third terms of the equation represent contributions from newly forming and preexisting CCPs, respectively. I further propose that cooperative activation of PIP5K arises within the confined environment of newly forming CCPs rather than through direct conformational changes. Cooperative effects were modeled using Hill equations, with coefficients (n and m) representing newly forming and preexisting CCPs. Parameter tuning allowed the model to reproduce the modest 2–3-fold increase in kinase activity

observed experimentally. As the Hill coefficient increased, basal PIP₂ levels rose and reached a plateau (Figure 1C, inset).

The model also recapitulated the hump phenotype under conditions of β -arrestin knockdown. At high Hill coefficients (n and $m = 6$), PIP₂ recovery remained incomplete and exhibited a hump-like trajectory (Figure 2A). Correspondingly, PIP5K activation was delayed and sustained at higher levels due to cooperative activation of the kinase (Figure 2B). Knockdown of β -arrestins further prolonged this delay, producing a slow recovery phase followed by accelerated restoration of PIP₂. These results suggest that the hump arises from delayed, cooperative clustering of PIP5K mediated by residual β -arrestin-dependent CCP formation.

We next dissected the relative contributions of preexisting and newly forming CCPs by varying constants a and b (Figure 3A). When $a = 0$, eliminating the contribution of newly forming CCPs, PIP₂ recovery was substantially reduced, more so than when $b = 0$. This indicates that newly forming CCPs play the dominant role in PIP₂ homeostasis during desensitization of PAR2. Moreover, the delayed activation pattern of PIP5K disappeared when $a = 0$ but persisted when $b = 0$ (Figure 3B), further highlighting the central role of newly forming CCPs in shaping lipid kinase dynamics. As shown in Figures 3C and D, the PIP₂ hump and PIP5K activity were reproduced by $b = 0$ which reflects that only newly forming CCP works for the recovery, suggesting that in the condition, newly forming CCP can be a key locus of the PIP₂ recovery. However, it cannot be ruled out the contribution of preexisting CCP due to low degree of PIP5K activation and PIP₂ recovery.

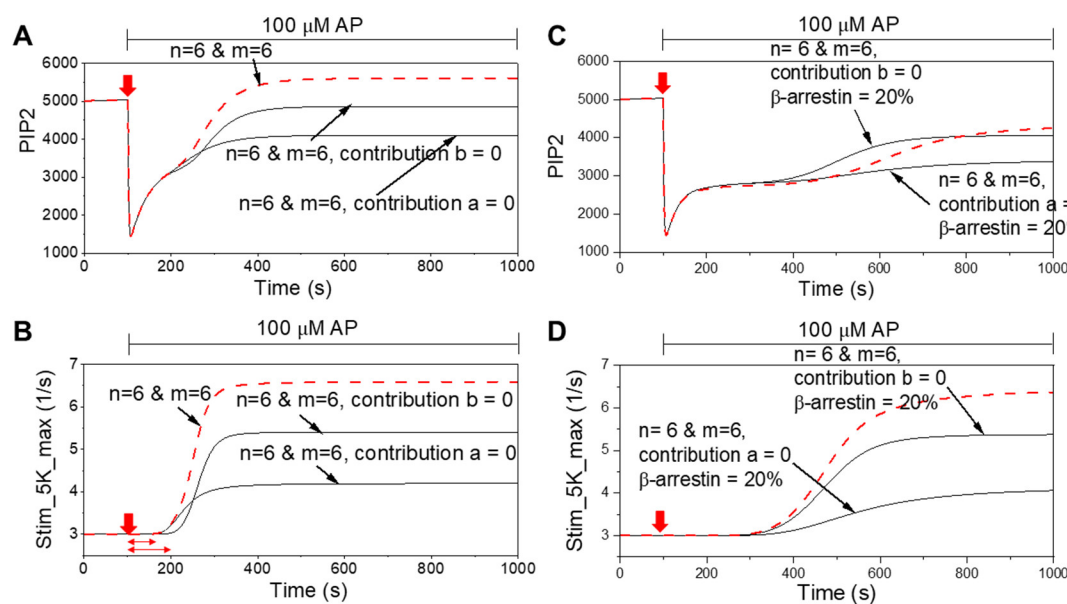


Figure 3. Newly forming clathrin-coated pits (CCPs) make the dominant contribution to PIP₂ recovery. (A) Simulated PIP₂ recovery under conditions where contribution constants were set to zero. Setting $a = 0$ (eliminating the contribution of newly forming CCPs) markedly impaired PIP₂ recovery, whereas setting $b = 0$ (eliminating the contribution of preexisting CCPs) produced a smaller effect. The red broken lines in A & B indicate the level of PIP₂ and PIP5K activity in control condition (n & $m = 6$). (B) PIP5K activity, represented by *stim_5K_max*, as a function of CCP contribution. Loss of newly forming CCP input ($a = 0$) strongly reduced and delayed kinase activity, while loss of preexisting CCP input ($b = 0$) had a relatively marginal effect. These relative constants were optimized to produce marginal increase of PIP5K activity and hump formation during PIP₂ recovery induced by β -arrestin knock-down. (C-D) The effect of removal of a or b contribution during β -arrestin knockdown in PIP₂ and PIP5K activity. The red broken lines in C & D indicate the level of PIP₂ and PIP5K activity during β -arrestin knockdown.

Interestingly, constraining $m = 1$ to reflect the lack of cooperative PAR2- β -arrestin clustering within preexisting CCPs had some effect on either PIP₂ recovery or PIP5K activation kinetics (Figure 4). These results indicate that cooperativity associated with newly forming CCPs is the primary

contributor to PIP₂ regeneration, but not full contributor as previously mentioned, suggesting that the contribution between preexisting and newly forming CCP on the cooperative behavior of the kinase would be balanced by the Hill coefficients (n and m) and weighting constants (a and b) in the individual cases.

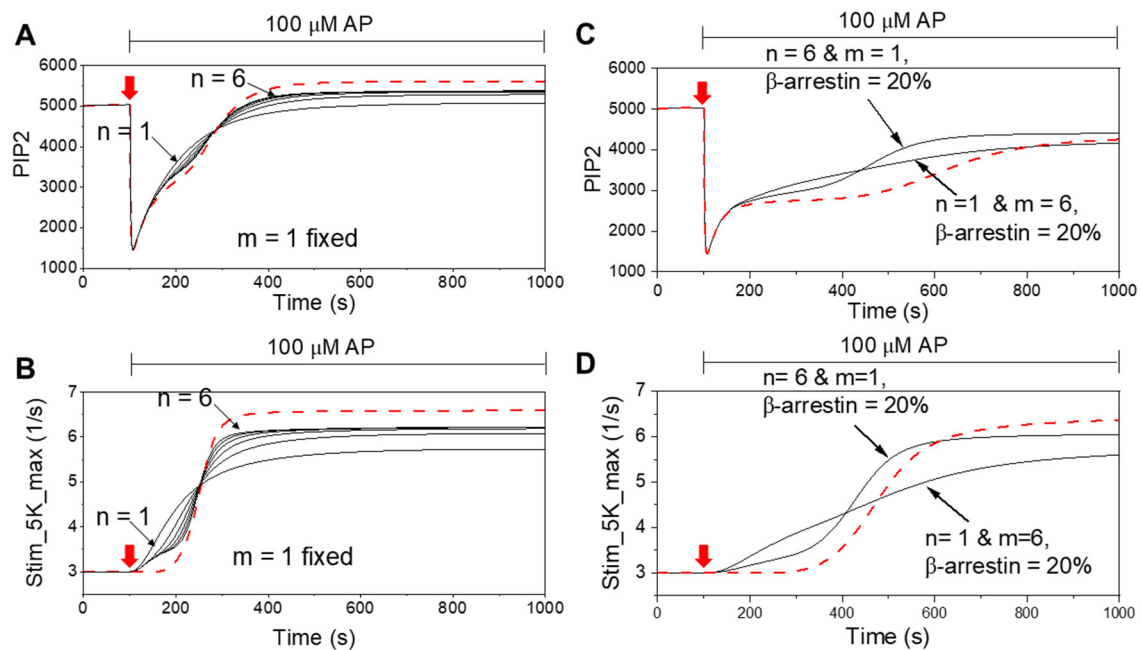


Figure 4. Both preexisting and newly forming CCPs contribute to PIP₂ recovery. (A) Simulated PIP₂ hydrolysis and recovery after PAR2 activation (100 μ M AP) under varying cooperative constants. The cooperative constant for preexisting CCPs was fixed at $m = 1$, while the constant for newly forming CCPs (n) was varied from 1 to 6. Increasing n produced progressive enhancement of PIP₂ recovery, with $n = 6$ at $m = 1$ yielding a recovery profile only ~60% compared with the condition where both n and m were set to 6 (red broken line). (B) PIP5K activity, represented by *stim_5K_max*, as a function of CCP contribution fixed at $m = 1$. The cooperativity of the kinase activity was not only from newly forming CCP but also from preexisting CCP. (C-D) The cooperativity of newly forming CCP can produce hump of PIP₂ recovery by β -arrestin knockdown. The red broken lines in C & D indicate the level of PIP₂ and PIP5K activity during β -arrestin knockdown when the n and $m = 6$ were set.

4. Discussion

This study identifies a cooperative mechanism by which transient β -arrestin-dependent activation of PIP5K restores PIP₂ levels at the plasma membrane following activation of G_q-coupled receptors such as PAR2. Through mathematical modeling, we show that even modest increases in PIP5K activity—amplified by cooperative interactions within CCPs—are sufficient to regenerate PIP₂ and maintain receptor signaling competence. This mechanism provides a molecular explanation for the long-standing paradox of how G_qPCRs can undergo endocytosis despite hydrolysis of their essential substrate, PIP₂.

4.1. Cooperative PIP5K Activation as the Key Regulatory Principle

My model demonstrates that the recovery of PIP₂ cannot be explained by linear enzyme activation alone. The inclusion of Hill-type cooperative behavior in PIP5K activity, mediated by β -arrestin-GPCR complexes, reproduces the experimentally observed “hump” pattern of PIP₂ recovery following β -arrestin knockdown. The Hill coefficients (n and m) describe the degree of cooperativity within newly forming and preexisting CCPs, respectively. High cooperativity (n & $m \approx 6$) in CCPs accounts for both the overshoot in PIP₂ recovery and the delayed activation kinetics seen experimentally. Importantly, these Hill coefficients should be viewed as phenomenological parameters that capture emergent cooperative behavior rather than literal molecular stoichiometry.

Such cooperativity likely arises from multiple weak and transient interactions among β -arrestin, GPCRs, and endocytic adaptors within spatially confined CCP microdomains. How about effect of contribution constants a (newly forming CCP) and b (preexisting CCP) on the PIP₂ recovery? Probably, the relative contribution can be determined by ratio of the newly forming CCP and preexisting CCP depending on cell cycle and classes of G_qPCR.

4.2. Functional Difference Between Newly Forming and Preexisting CCPs

By systematically varying contribution parameters (a and b), our simulations revealed that newly forming CCPs are the primary drivers of delayed PIP₂ regeneration with nonlinearity, whereas preexisting CCPs play a marginal role for the dynamics. Newly forming CCPs provide an environment where β -arrestin–receptor complexes can nucleate adaptor proteins and clathrin assembly, enabling cooperative clustering of PIP5K which makes delayed kinetics. In contrast, preexisting CCPs are diffusion-limited and recruit the GPCR- β -arrestin complexes at the edge of the CCPs, restricting the formation of cooperative assemblies which make linear saturation kinetics. This distinction explains why β -arrestin–dependent clustering at newly forming CCPs produces robust recovery kinetics, whereas preexisting CCPs cannot fully compensate under knockdown conditions.

4.3. Broader Implications of Transient and Weak Interactions

Beyond this specific system, our findings highlight the broader principle that weak and transient protein–protein interactions can collectively generate robust signaling outcomes. Although individual β -arrestin–PIP5K interactions are short-lived, their repeated and cooperative engagement within confined CCPs amplifies enzymatic activity and ensures lipid homeostasis. This concept aligns with emerging views that weak, dynamic assemblies—rather than stable complexes—underlie efficient information transfer in signaling networks. Similar cooperative mechanisms may regulate other lipid kinases or scaffolded enzymes operating in nanoscale membrane domains.

Transient protein–protein interactions can induce weak or short-lived conformational changes in kinases that enhance their catalytic output without fully stabilizing the active conformation. Such partial structural rearrangements, often involving local loop motions or small domain shifts, can transiently align catalytic residues or promote substrate accessibility, thereby producing a modest but physiologically significant increase in activity [22]. This contrasts with stable conformational changes, such as strong scaffold binding, which lock the enzyme into a fully active state and yield maximal catalytic efficiency [23]. The transiently induced state thus represents a dynamic intermediate between inactive and fully active conformations—sufficient to fine-tune signaling output while maintaining reversibility and rapid responsiveness to fluctuating stimuli. In this framework, transient binding events act as kinetic modulators that bias the conformational ensemble toward catalytically permissive states without requiring full structural rearrangement, allowing cells to modulate kinase activity in a graded, energy-efficient manner.

4.4. Potential Crosstalk of PAR2–PIP₂–PIP5K Signaling with Oncogenic KRAS

Our findings describing transient β -arrestin–dependent activation of PIP5K and cooperative recovery of PIP₂ at the plasma membrane have potential implications beyond receptor endocytosis. Increasing evidence suggests that PAR2-mediated lipid signaling intersects with KRAS activation and phosphoinositide 3-kinase (PI3K)–Akt pathways, all of which play central roles in cancer progression. Protease activated receptors are aberrantly expressed in many cancers, including breast, pancreatic, and colorectal tumors, where it enhances proliferation, migration, and epithelial–mesenchymal transition [24,25]. KRAS requires stable association with the inner leaflet of the plasma membrane via electrostatic interactions between its C-terminal polybasic domain and negatively charged phosphoinositide, particularly phosphatidylserine (PS) and PIP₂ [26]. Thus, transient PIP₂ regeneration following PAR2 activation may reinforce KRAS membrane anchoring and preserve its signaling-competent conformation, providing a mechanistic bridge between protease sensing, lipid

metabolism, and Ras activation. Once activated, KRAS recruits and activates PI3K, which phosphorylates PIP₂ to generate PIP₃, leading to Akt recruitment toward the plasma membrane and the initiation of downstream survival signaling [27,28]. Through this cooperative loop, PAR2 signaling could synergize with oncogenic KRAS to sustain PI3K–Akt activity, enhancing tumor cell proliferation, motility, and resistance to stress.

Taken together, the PAR2–PIP₂–KRAS–PI3K–Akt axis may represent a molecular hub of cooperative signaling that links receptor desensitization, lipid regeneration, and oncogenic amplification. The cooperative PIP5K activation described in our model provides a plausible kinetic framework for how weak, transient β -arrestin–PIP5K interactions can amplify lipid-dependent signaling in cancer contexts. While each interaction is short-lived, their cumulative effect within spatially confined domains produces a sustained output—analogueous to how stochastic, diffusion-limited binding can yield emergent cooperativity. In tumor cells, where PIP5K and PAR2 are often overexpressed, this mechanism could lower the activation threshold for KRAS–PI3K–Akt signaling, enabling persistent survival and invasive behavior even in fluctuating extracellular conditions.

4.5. Limitations and Outlook

While the present model successfully reproduces key kinetic features of PIP₂ recovery, it simplifies several aspects of the system. It does not explicitly account for protein diffusion barriers, PIP5K isoform specificity, or dynamic exchange of PIP5Ks within CCPs. Future experimental studies employing single-molecule imaging, optogenetic control of PIP5K, and real-time FRET biosensors could quantitatively test these predictions and reveal the spatiotemporal organization of these transient complexes. Incorporating stochastic spatial or agent-based models will further refine our understanding of how nanoscale cooperativity translates into large-scale lipid regulation.

5. Conclusions

In conclusion, our results demonstrate that transient β -arrestin-dependent activation of PIP5K, though modest in magnitude, is sufficient to restore PIP₂ levels through cooperative regulation within CCPs. This cooperative mechanism not only explains the paradox of PIP₂-dependent endocytosis during G_qPCR activation but also establishes a broader paradigm: cellular homeostasis and signaling fidelity emerge from the collective effects of dynamic, weak interactions that fine-tune enzymatic and trafficking processes.

Author Contributions: S.R.J. designed simulations with Virtual Cell and wrote this manuscript.

Data Availability Statement: mathematical modeling would be open source in the Virtual Cell system.

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Conflicts of Interest: No conflict with JSR Biomedical Science Consulting LLC.

Abbreviations

PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P ₂)

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