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A New Mode of Ca^{2+} Signaling by G Protein-Coupled Receptors: Gating of IP_3 Receptor Ca^{2+} Release Channels by Gβγ

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Summary

The most common form of Ca^{2+} signaling by Gq-coupled receptors entails activation of PLCβ2 by Gαq to generate IP_3 and evoke Ca^{2+} release from the ER [1]. Another form of Ca^{2+} signaling by G protein-coupled receptors involves activation of Gi to release Gβγ, which activates PLCβ1 [2]. Whether Gβγ has additional roles in Ca^{2+} signaling is unknown. Introduction of Gβγ into cells activated Ca^{2+} release from the IP_3 Ca^{2+} pool and Ca^2 oscillations [3, 4]. This can be due to activation of PLCβ1 or direct activation of the IP_3R by Gβγ. We report here that Gβγ potently activates the IP_3 receptor. Thus, Gβγ-triggered $[Ca^{2+}]_i$ oscillations are not affected by inhibition of PLCβ. Coimmunoprecipitation and competition experiments with Gβγ scavengers suggest binding of Gβγ to IP_3 receptors. Furthermore, Gβγ inhibited IP_3 binding to IP_3 receptors. Notably, Gβγ activated single IP_3R channels in native ER as effectively as IP_3. The physiological significance of this form of signaling is demonstrated by the reciprocal sensitivity of Ca^{2+} signals evoked by Gi- and Gq-coupled receptors to Gβγ scavenging and PLCβ inhibition. We propose that gating of IP_3R by Gβγ is a new mode of Ca^{2+} signaling with particular significance for Gi-coupled receptors.

Results and Discussion

Gβγ-Evoked Ca^{2+} Release Is Independent of PLC Activation

In a previous work we reported that infusion of Gβγ into pancreatic acinar cells evoked two types of $[Ca^{2+}]_i$ oscillations: high-frequency, short-lasting spikes and low-frequency, longer-lasting Ca^{2+} spikes (see also Figures 1G and 1H). These effects were blocked by two Gβγ scavengers, the Gβγ binding domain of the β-adrenergic receptor kinase 1 (βARK1) and a mutant Gαi (G203A), as well as by heparin, a competitive inhibitor of the IP_3R [4]. These findings were attributed to activation of PLCβ1 by Gβγ. However, the present findings refute this interpretation. In pancreatic acini, one can reliably estimate $[Ca^{2+}]_i$ by measuring the Ca^{2+}-activated Cl^- current, which reflects Ca^{2+} spiking at the apical pole [5]. With such measurements, two independent protocols indicate that Gβγ activated Ca^{2+} release independent of PLCβ activation. U73122, an inhibitor of all PLCβ isoforms, abolished $[Ca^{2+}]_i$ oscillations evoked by stimulation of the Gq-coupled M3 receptor with a low concentration of carbachol, and it largely inhibited the sustained response evoked by 1 mM carbachol (Figure 1A). We could still induce maximal Ca^{2+} release in the presence of U73122 by using the Ca^{2+} ionophore ionomycin. By contrast, U73122 failed to inhibit $[Ca^{2+}]_i$ oscillations induced by Gβγ (Figures 1B and 1C). Gβγ-induced $[Ca^{2+}]_i$ oscillations were observed in 16 out of 19 control cells and, similarly, in 5 out of 6 cells treated with U73122. Comparable results were obtained with an anti-PIP2 antibody, another inhibitor of PLCβ-mediated Ca^{2+} signaling. This anti-PIP2 antibody has been demonstrated to specifically inhibit several PIP2-dependent actions [6]. Because antibody diffusion into cells from a patch pipette is slow, its effects were expected to be time dependent, as observed (Figures 1D and 1E). After 200 and 400 s of infusion into the cells, the responses to maximal stimulation of Gq-coupled M3 and bombesin receptors were blocked by about 60% and close to 95%, respectively (Figure 1F). Nevertheless, the anti-PIP2 antibody failed to block the responses to Gβγ, even after 20 min of infusion, while inhibiting the responses to carbachol in the same cells (Figures 1G and 1H). A similar lack of effects of anti-PIP2 antibody on the responses to Gβγ were observed in 6 out of 8 cells.

The results in Figure 1, showing that inhibition of PLC activity by two different inhibitors is without effect on Gβγ-activated Ca^{2+} signaling, suggest that Gβγ can activate Ca^{2+} release from internal stores independently of IP_3 production. Below we show that this is due to activation of the IP_3R Ca^{2+} release channel by Gβγ.

Gβγ Interacts with IP_3 Receptors

Interaction between Gβγ and IP_3Rs was examined by coimmunoprecipitation (co-IP) with cerebellar extracts, a rich source of IP_3R1 and Gβγ. The low abundance of IP_3Rs precluded comparable experiments in pancreatic acini, in particular the IP_3 binding assays. Cerebellar extracts were fractionated on a linear sucrose gradient to isolate fractions enriched in both proteins (Figure 2A, fractions 8–11). In reciprocal IP, both proteins were found to interact (Figures 2B and 2C). Notably, the Gβγ scavengers βARK and Gαi(G203A) prevented the co-IP. Similarly, a peptide (βARKP) that constitutes the Gβγ binding motif of βARK and inhibits binding of Gβγ to target proteins [7, 8] prevented the co-IP. These findings are consistent with dissociation of the IP_3R1-Gβγ complex by these agents. The significance of these findings is that (1) the interaction of Gβγ with the IP_3R1 is specific and (2) the binding of Gβγ to IP_3R1 is not tight because

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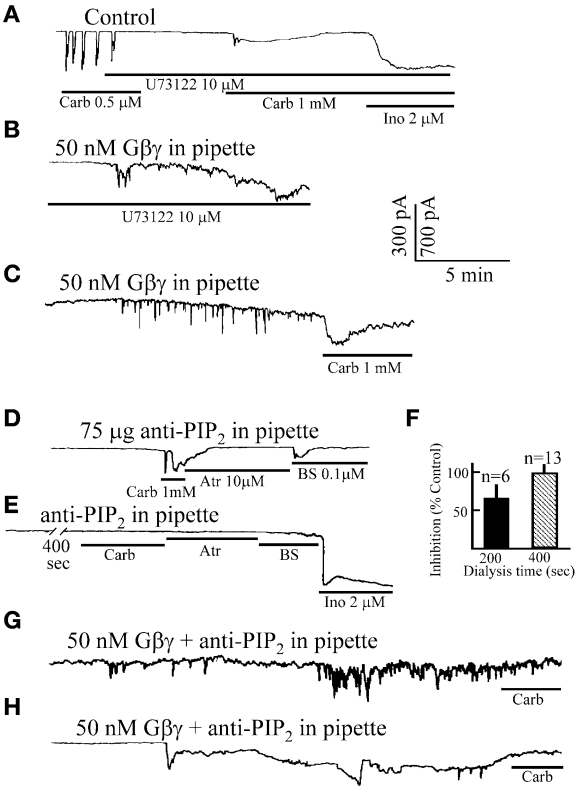

Figure 1. Gβγ-Activated $[Ca^{2+}]_i$ Oscillations Are Independent of PLCβ Activity

Whole-cell Ca^{2+}-activated Cl^- current was used for following changes in $[Ca^{2+}]_i$. Rat pancreatic acinar cells were infused with a control pipette solution (A), pipette solution containing 50 nM Gβγ (B, C, G, H), or pipette solution containing 75 μg/ml anti-PIP2 antibody (D–F). In (A) and (B), the cells were treated with 10 μM U73122, as indicated. Scale bars indicate the period of exposure to 0.5 μM or 1 mM carbachol (carb), 0.1 μM bombesin (BS), 2 μM ionomycin (Ino), or 10 μM atropine (Atr). In all experiments, recording commenced about 15 s after the whole-cell configuration was established.

it can be competed away by two proteins that bind Gβγ and it was disrupted by a peptide that interferes with binding of Gβγ to target proteins.

The majority of Gβγ was retained at the top of the gradient, whereas most of the IP_3R1 migrated to the bottom (Figure 2A). The separation of IP_3R1 from Gβγ allowed us to study the effects of Gβγ on a vital IP_3R function, that of IP_3 binding. IP_3 binding to fraction 4 (containing only Gβγ) was used as a control. IP_3 binding to IP_3R1 in fraction 10, which contains both IP_3R and Gβγ, was low (Figure 2D). Notably, binding was increased about 5-fold by the Gβγ binding inhibitor βARKP (Figure 2D) in a concentration-dependent manner (Figure 2E), as well as by the Gβγ scavenger βARK. By contrast, Gβγ had no further effect on IP_3 binding. Reciprocal effects on IP_3 binding to the IP_3R1 were observed in fraction 15, which contained IP_3R but lacked Gβγ. Thus, βARKP and βARK had no effect on IP_3 binding, whereas Gβγ inhibited it in a concentration-dependent manner. These findings suggest that Gβγ modulates IP_3 binding to the IP_3 receptors. To determine the state of the native IP_3Rs with respect to Gβγ binding, we measured the effects of βARKP and Gβγ on IP_3 binding to IP_3Rs in cerebellar microsomes. Figure 2E shows that βARKP had no effect, whereas Gβγ inhibited IP_3 binding, suggesting that most native IP_3Rs are not associated with Gβγ.

Gβγ Activates the IP_3 Receptors Ca^{2+} Release Channel

Gβγ could inhibit IP_3 binding to IP_3Rs by allosteric modification of the IP_3 binding site or by competing with IP_3 for binding to the same site/region of the IP_3R1. To study the significance of this modulation, we examined the effects of purified recombinant Gβγ on the single-channel activity of native IP_3 receptors recorded in the nuclear envelope of *Xenopus* oocytes. Normally, channel gating is not observed unless the patch pipette contains IP_3 (Figure 3). Remarkably, Gβγ activated channel gating in the absence of added IP_3. Gβγ elicited channel activity as efficaciously as IP_3 (Figure 3C) and to comparable levels of channel-open probability (Figure 3F). The addition of Gαi(G203A) to scavenge Gβγ eliminated its ability to activate channel gating (Figure 3E), demonstrating the specificity of its effect. Furthermore, channels activated by Gβγ were inhibited by heparin (Figure 3D), a competitive IP_3R channel inhibitor [4]. Electrophysiological demonstration that Gβγ activates channel gating, together with biochemical evidence that Gβγ inhibits IP_3 binding, indicates that Gβγ interacts with IP_3Rs and stabilizes a channel conformation that exhibits activity comparable to that of the IP_3 bound channel. These results therefore suggest that IP_3 and Gβγ binding represent two alternative modes of activating IP_3 receptors.

Ca^{2+} signaling evoked by Gq-coupled receptors is dependent on αq-mediated activation of PLCβ, whereas Ca^{2+} signaling evoked by Gi-coupled receptors is mediated by Gβγ [9]. Therefore, to examine the physiological significance of Gβγ gating of IP_3 channels, we compared the effects of inhibiting PLCβ or scavenging Gβγ on Ca^{2+} signaling evoked by stimulation of Gq- and Gi-coupled receptors in the same cells with carbachol and vasoactive intestinal peptide (VIP), respectively. Activation of the Gs-coupled VIP receptors was shown to evoke Ca^{2+} spiking in pancreatic acini that can be mimicked by an increase in cytoplasmic cAMP [10]. In subsequent work we showed that although the M3 receptors activate Gαq [4], the VIP receptors use a Gs/Gi switching mechanism [11] to activate Gi and promote the release of Gβγ that is essential for Ca^{2+} signaling by these receptors [12]. For these experiments, we employed U73122 to inhibit PLCβ so that we could compare the effects of VIP on IP_3 production and Ca^{2+} signaling. Inhibition of PLCβ with U73122 (0.2 or 0.5 μM) inhibited or reduced the Ca^{2+} signaling response to 0.5 μM or 100 μM carbachol, respectively, as expected, whereas it had minimal effects on the Ca^{2+} signals evoked by VIP (Figures 4A and 4B). U73122 similarly inhibited the carbachol response in cells that had not been treated with VIP or exposed to U73122 for a long period of time (Figure 4C). Reciprocal observations were made in cells infused with as little as 20 nM of the Gβγ scavenger Gαi (G203A), which abolished the response to VIP without affecting

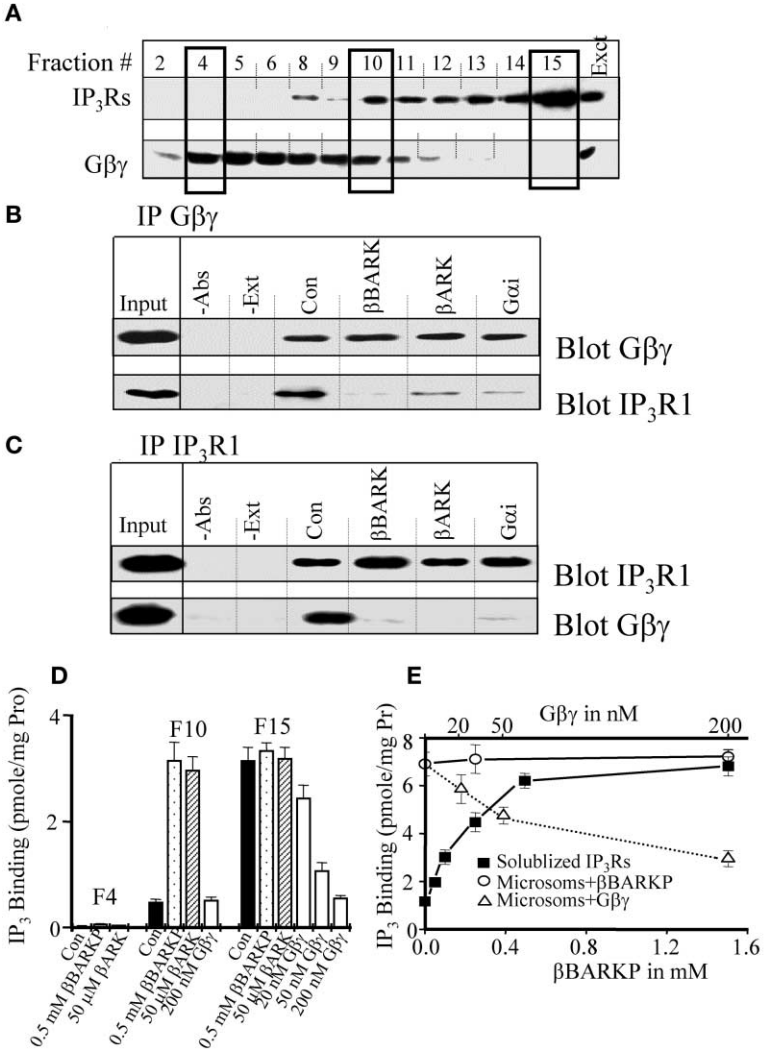

Figure 2. Co-IP of IP_3R1 and Gβγ and Effect of Gβγ on IP_3 Binding to IP_3R1

(A) Blot of $IP_3R1$and Gβγ in 15 fractions collected from a linear sucrose gradient. Fractions similar to 8–11 were pooled and used for immunoprecipitating Gβγ (B) or IP_3R1 (C). The pooled extracts were incubated with buffer (Control, Con); 0.5 mM βARKP, 50 μM βARK, or 200 nM Gαi(G203A) (Gαi) for 30 min at 0°C before addition of antibodies and beads. For controls, in the first lane in each case the primary antibodies were omitted (−Abs), and in the second lane the extract was omitted (−Ext). The input represents 13% of the material used for the IP. Fractions 4, 10, and 15 were used for the IP_3 binding assay in panel (D). The extracts from each fraction were added to incubation medium containing the indicated final concentrations of Gβγ, βARKP, and βARK and incubated for 30 min at 0°C before addition of ^3H-IP_3 and a further incubation for 15 min at 0°C. At the end of the incubations, the solubilized IP_3R1 was precipitated with ethylene glycol, and binding of IP_3 was determined. Similar experiments were performed with fraction 10 from a separate gradient and [βARKP] between 0.05 and 1.5 mM. The dose response for βARKP-mediated increase in IP_3 binding is plotted in panel E (squares). Intact cerebellar microsomes were used to test the effect of βARKP (circles) and Gβγ (triangles) on IP_3 binding to IP_3R1 in native membranes. The behavior of IP_3R1 in cerebellar microsomes was similar to that in fraction 15, indicating that the IP_3R1 in isolated cerebellar microsomes is largely free of Gβγ and that Gβγ can regulate the IP_3R1 in native membranes.

the responses to either low or high concentrations of carbachol (Figure 4D). It was necessary to increase the concentration of Gαi (G203A) to 50 (Figure 4E) and 200 nM (Figure 4F) to inhibit the responses to 0.5 μM and 100 μM carbachol, respectively. These findings were corroborated by measurements of IP_3 production in cells stimulated by the two agonists. VIP failed to elicit a measurable increase in IP_3, in agreement with findings with other native Gi-coupled receptors (Figure 4G), whereas 0.5 μM carbachol elicited a readily measurable increase that was inhibited by 0.5 μM U73122. The same concentration of U73122 also partially inhibited IP_3 production in response to maximal stimulation with 100 μM carbachol. Taken together, these results highlight the two modes of signal transduction by GPCR and their differential dependence on IP_3 production and Gβγ. These results provide physiological support for the idea that Ca^{2+} signals elicited by Gi-coupled receptors, although mediated by Ca^{2+} release through the IP_3Rs, are promoted by IP_3R activation by Gβγ.

The combined findings of the present work indicate that Gβγ activates IP_3Rs. The IP_3R is the third channel known to be regulated by Gβγ, the others being the inward-rectifying K^+ channels and the L-type Ca^{2+} channels [13]. Gβγ regulates the K^+ and Ca^{2+} channels by directly binding to these channels [13]. The observed co-IP of Gβγ and IP_3R and inhibition of IP_3 binding to solubilized IP_3Rs by Gβγ are consistent with such direct regulation.

The present work expands the modes by which G proteins can activate Ca^{2+} signaling. Thus, GPCRs can activate Ca^{2+} signaling by activation of PLCβ to generate IP_3 or by activation of IP_3Rs with Gβγ. Inhibition of all forms of Ca^{2+} signaling by the Gβγ scavengers and inhibitors (Figure 4 and [4]) suggests that Gβγ participates in both Gq- and Gi-dependent Ca^{2+} signaling. However, activation of IP_3Rs by Gβγ appears to be of particular importance for Gi-coupled receptors. Activation of these receptors releases Gαi and Gβγ. Although it is generally assumed that Gβγ activates PLCβ1 to trigger a Ca^{2+} signal [9], it is noteworthy that it is difficult to detect any IP_3 production by activation of Gi-coupled receptors, as illustrated here for the VIP receptor (Figure 4). Our results suggest that an alternative explanation is that Gβγ directly activates IP_3Rs, independently of IP_3 production. Indeed, the Ca^{2+} signals evoked by VIP

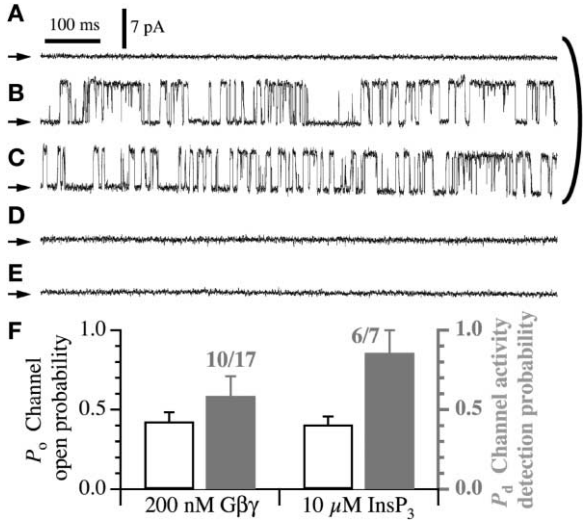

Figure 3. IP_3-Independent Gating of IP_3R1 by Gβγ

Typical patch-clamp current traces from outer membrane patches obtained from isolated *Xenopus* oocyte nuclei. Arrows indicate the closed-channel current level. (A) The pipette solution contained 0.02% lubrol; no channel activity was observed in any of 18 patches. (B) The pipette solution contained 200 nM Gβγ and 0.02% lubrol; $InsP_3R$ channel activities were observed in ten of 17 patches. (C) The pipette solution contained 10 μM $InsP_3$; $InsP_3R$ channel activities were observed in six of seven patches. Current traces (A)–(C) (enclosed with the brace) were recorded in membrane patches obtained from the same region of the same oocyte nuclei. (D) Pipette solution contained 200 nM Gβγ, 100 mg/ml heparin, and 0.02% lubrol; no channel activity was observed in any of 12 patches. In the same region of the same oocyte nuclei, $InsP_3R$ channel activities were observed in eight of 11 patches with pipette solution containing 10 μM $InsP_3$. (E) Pipette solution contained 200 nM Gβγ, 500 nM Gαi(G203A), and 0.02% lubrol; no channel activity was observed in any of five patches. In the same region of the same oocyte nuclei, $InsP_3R$ channel activities were observed in nine of ten patches with pipette solution containing 10 μM $InsP_3$. (F) Channel-open probability (P_o, open bars) and channel activity detection probability (P_d = number of membrane patches exhibiting $InsP_3R$ channel activities/total number of membrane patches obtained, filled bars) for pipette solutions containing 200 nM Gβγ and 0.02% lubrol, or 10 μM $InsP_3$. P_o and P_d in the presence of the two agonists exhibit no statistically significant differences ($p > 0.05$).

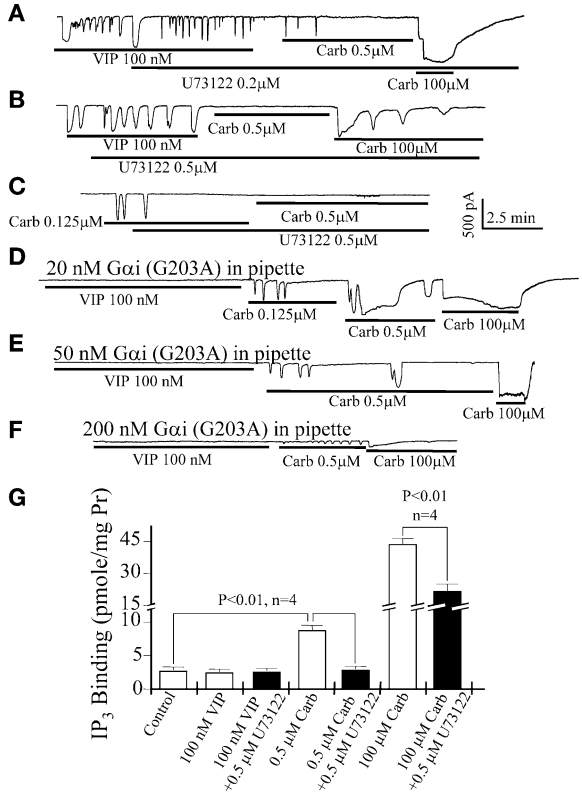

Figure 4. Differential Sensitivity of Gi- and Gq-Dependent Ca^{2+} Signaling to Inhibition of PLCβ and Gβγ

Mouse pancreatic acini were dialyzed with control pipette solution (A–C) or pipette solution containing 20 nM (D), 50 nM (E), or 200 nM (F) Gαi(G203A) for 7 min prior to stimulation with the indicated concentrations of VIP and carbachol as indicated by the bars. In (A), after the control period for recording the response to VIP, the cells were exposed to 0.2 μM U73122 for the remainder of the experiment. In (B), the cells were exposed to 0.5 μM U73122, and in panel (C) oscillations similar to those evoked by VIP were induced with 0.125 μM carbachol before the exposure to 0.5 μM U73122. Note that in mouse pancreatic acini 0.5 μM carbachol generated a robust Ca^{2+} signal (D). Similar results to those in panels (A)–(F) were obtained in at least three experiments. Panel (G) shows results of two experiments performed in duplicates to measure IP_3 production by cells stimulated with the indicated concentrations of agonist. A portion of the cells was treated with 0.5 μM U73122 for 2 min (filled columns) prior to stimulation with agonists. The results are the mean ± S.E.M of the four determinations.

stimulation were exquisitely sensitive to scavenging Gβγ with Gαi (G203A), and at the same time they were not inhibited by concentrations of the PLCβ inhibitor U73122 that inhibited a more intense signals evoked by the Gq-coupled M3 receptor in the same cells. Hence, we propose that a major mechanism to evoke Ca^{2+} signaling by Gi-coupled receptors is direct activation of IP_3Rs by Gβγ.

Activation of PLCβ and IP_3Rs by Gβγ is not mutually exclusive. Gβγ appears to activate the two proteins with similar potency. Half maximal activation of all PLCβ isoforms requires 100 nM or higher Gβγ [9]. This value is in the same range as the concentration that markedly activated the IP_3R (200 nM Gβγ). This would suggest that the mode of organization of the signaling complex will determine the preferential pathway used by the Gi-coupled receptors to activate Ca^{2+} signaling. Alternatively, the two mechanisms may be used in series to initiate a Ca^{2+} signal: local activation of IP_3Rs with Gβγ and a more distal activation of Ca^{2+} release by IP_3. Such a mechanism may also contribute to Ca^{2+} signaling by Gq-coupled receptors. Whereas IP_3 can diffuse rapidly in the cytosol [14], diffusion of Gβγ is very limited [3]. Therefore, Gβγ would be ideal for local activation of IP_3Rs most adjacent to the plasma membrane. Cell signaling, including Ca^{2+} signaling [15], occurs by the assembly of protein complexes in cellular microdomains, such as caveolae [16], the post-synaptic densities [17], and the luminal pole of epithelial cells [15]. In the case of Ca^{2+} signaling, the complexes include receptors, G proteins, IP_3Rs, and Ca^{2+} pumps [15], and they are the sites from which Ca^{2+} waves initiate near the plasma membrane [5, 18–20]. Receptor-triggered release of Gβγ within these complexes could initiate and/or facilitate

activation of the sub-set of IP_3Rs that function as a trigger pool to launch $[Ca^{2+}]_i$ oscillations and Ca^{2+} waves.

In summary, the present work provides evidence for IP_3-independent regulation of IP_3Rs by $G\beta\gamma$. The widespread expression of G proteins and GPCRs suggests that this alternative mode of activation of the IP_3Rs is a general mechanism present in all cells. Physiologically, it appears that activation of the IP_3 receptors by $G\beta\gamma$ is central to Gi-coupled receptor-generated Ca^{2+} signals.

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