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COMMENTARY

Modes of Interactions between Signaling Pathways

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ABSTRACT. The study of signaling pathways has begun to uncover the mechanism by which cells respond and adapt to extracellular stimuli. It has become increasingly clear that the signaling pathways interact with one another to form a complex network through which regulation occurs. Here, we focus on three mechanisms by which signaling pathways interact and the physiological consequences of these interactions. Coincident signaling in long-term depression of synaptic responses in the cerebellum, protein kinase A gating of Ras to mitogen-activated protein kinase signal flow in proliferative responses, and a modified gating mechanism by phosducin resulting in feedback regulation of signal flow from rhodopsin to the cGMP phosphodiesterase in retinal light adaptation are analyzed as examples of different types of interactions between signaling pathways. These interactions allow the cell to spatially and temporally integrate complex information and respond in an appropriate and defined manner. BIOCHEM PHARMACOL **55**;9:1347–1352, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. signaling pathway interactions; coincidence signals; gates; feedback regulation

Every living organism must respond and adapt to stimuli in an organized manner in order to survive within its environment. Responses to stimuli occur primarily at the cellular level, and intracellular machinery is responsible for the proper interpretation and processing of external stimuli. Generally, a cell must be able to assimilate information from its surroundings and internal milieu, and react in an appropriate and defined manner. Signal transduction pathways accomplish this by responding to a specific stimulus with a response whose nature, amplitude, and duration are predictable on the basis of the identity of the components that comprise the pathway. The proteins that participate in these signaling pathways can be organized into families, including receptors that receive extracellular signals [1]; transducers such as GTPases that connect the receptor to intracellular effectors [2]; adaptor proteins that serve as bidirectionally specific interfaces between receptors and transducers or effectors [3, 4]; and effectors such as second messenger producing enzymes, protein kinases, protein phosphatases, and ion channels. GTPases function through the binding and hydrolysis of GTP, which confers a cycle of activity in which downstream effectors are activated. Protein kinases propagate signals by phosphorylating effectors, which can alter subcellular location, enzyme activity, or the capability of proteins to interact with their targets [5, 6]. The phosphatases act directly opposite to the protein kinases through dephosphorylation of their effectors, likewise affecting protein function and/or activity [7]. Ion channels respond either to ligands extracellularly or to voltage across the cell membrane. The channels respond to these stimuli by then allowing the influx or efflux of charged ions, further potentiating or inhibiting signaling pathways. Through the use of these diverse signaling mechanisms, cellular responses can be both specific and predictable.

Typically, signaling pathways are depicted and studied as linear cascades. This is a necessary first step in understanding the mechanisms underlying signal transduction. It is also becoming more evident that within the cell, the different signaling pathways interact with and regulate one another. Interactions between signaling pathways increase the capability of a cell's processing potential, allowing for the integration of diverse information. This also enables a cell to respond to multiple simultaneous or sequential stimuli with concurrent adaptation and to "remember" which pathways have been activated and thus respond in an appropriate manner.

As our understanding of how individual signaling pathways work increases, different modes by which these signaling pathways can interact are also being recognized. In this article, we discuss three examples of interactions between signaling pathways. The first of these uses coincident detectors; the second involves gating, where one pathway regulates signal flow through another pathway; and the third example deals with a feedback loop that constitutes a modified gating phenomenon where a single signal travels through two distinct pathways to regulate the end point response. Coincidence detection is based on two distinct signaling pathways, A and B, converging on a single functional unit composed of one or more proteins known as the coincidence detector (Fig. 1A). The coincidence detector is able to recognize when the two converging pathways are activated within a set period of time of

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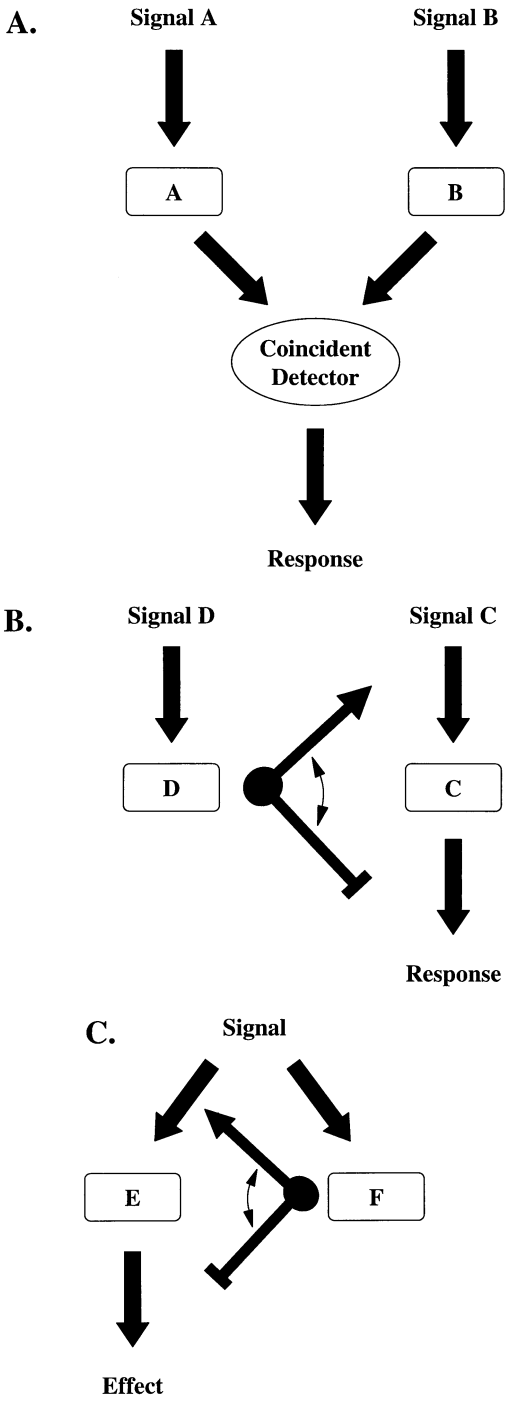

FIG. 1. Mechanisms by which signaling pathways may interact. (A) Two distinct pathways converge on a coincidence detector, which generates a response that is unique and different from that generated by each individual pathway. (B) A primary pathway that evokes a biological response is modulated by a second pathway through a gate, resulting in either inhibition (⊥) or stimulation (↓) of the primary pathway. (C) A single initial signal flows through multiple pathways, with one pathway regulating the other. Such regulation may be positive or negative.

one another. The detector then produces a unique response different from what is observed when either pathway is activated individually [8]. Thus, the coincident response can be functionally distinct or can be synergistic, i.e. greater than the sum of the responses to A and B. A coincidence detector enables the cell to produce a unique response only when specific pathways have been activated simultaneously or within a specific amount of time. Both signals are equally important, and it is not possible to recognize a hierarchy within the system. Specific isoforms of the effector enzyme adenylyl cyclase have been prominently described as coincident detectors [8–10].

Gating (Fig. 1B) is another mode by which two signaling pathways interact. Here, signal flow through the first pathway, C, is regulated by activation of a second pathway, D. Thus, two different signals interact in a hierarchical fashion [11, 12]. In a gated system, the response elicited is thus only modified, but not distinct from that which is evoked when pathway C is exclusively activated. Gating, therefore, gives a cell the ability to regulate responses based on the state of the cell, which, in turn, may be dependent on the signaling pathways that are activated. Both coincidence detection and gating are similar in that they allow two separate signals to activate different signaling pathways, which then act in a concerted fashion to enable the cell to respond to these stimuli in a specific manner.

Feedback (Fig. 1C) is a modified gating mechanism, which is unique in that it is dependent on only one initial signal. This signal can then modulate multiple pathways or activate a single pathway that regulates two or more downstream effectors. In such a system, one effector produces the biological effect and the other regulates the signal flow to the effector that produces this effect. This configuration enables a signaling system to adjust its sensitivity to the environment, preventing or potentiating signaling flow to the end-point response system. Although feedback regulation utilizes only one type of signal, it is responsive to sequential stimuli due to the change in sensitivity of the pathway by self-regulation.

COINCIDENT SIGNALING IN CEREBELLAR LONG-TERM DEPRESSION

Cerebellar LTD* is a type of synaptic plasticity in which prior activity results in a decreased response to stimuli. LTD is observed in several brain regions, including the hippocampus and cerebellum. In cerebellar LTD, synaptic inputs from both the parallel and climbing fibers converge on the sole cortical output cell, the Purkinje neuron. The synaptic inputs of the parallel and climbing fibers must be coincident, i.e. they must occur within a specified amount of time, in order for LTD to be induced. Ito *et al.* [13] and Sakurai [14] experimentally determined that both synaptic inputs must be present, as each alone did not induce LTD. The result of the simultaneous input is a depression in the efficacy of the parallel fiber synapse.

* *Abbreviations:* LTD, long-term depression; MAPK, mitogen-activated protein kinase; NO, nitric oxide; sGC, soluble guanylyl cyclase; cGMP, cyclic GMP; cAMP, cyclic AMP; ERK, extracellular-regulated kinase; PKA, protein kinase A; and MEK, mitogen-activated protein kinase-kinase.

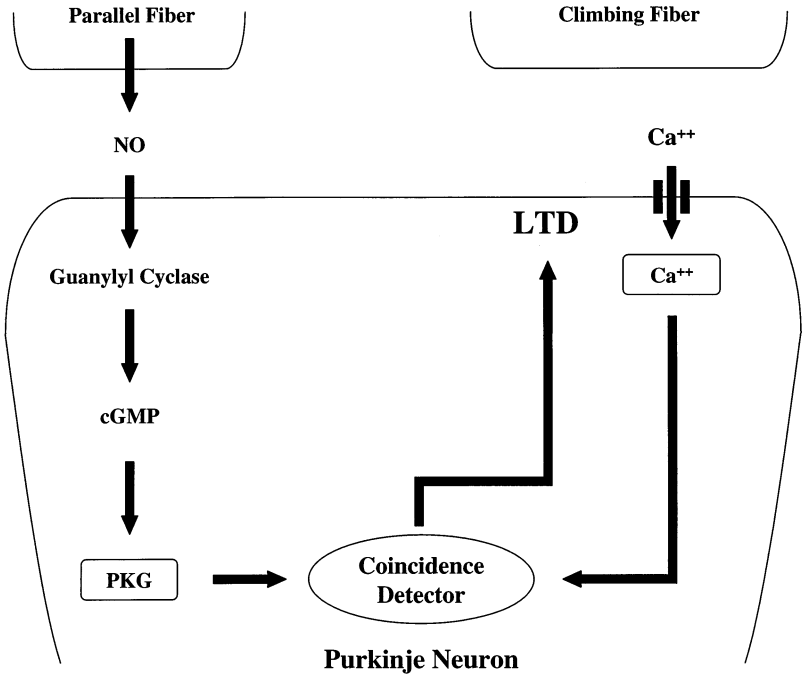

FIG. 2. Coincidence detection in cerebellar LTD. Release of NO from parallel fibers stimulates guanylyl cyclase in the Purkinje neuron, which results in an increase in cGMP and protein kinase G (PKG) activity. Stimulation of the climbing fiber results in elevation of intracellular Ca^{2+} in the Purkinje cell. Together, elevation of intracellular Ca^{2+} and PKG activate the coincidence detector. This results in LTD. The molecular identity of the coincident detector in the Purkinje neuron is currently unknown.

Investigation into how LTD is induced in the cerebellum has uncovered many of the cellular events that are required for LTD, as well as the key players and their roles in its induction. The climbing fiber axons that originate in the inferior olive make multiple synapses on the Purkinje neuron and are required solely as depolarizers of the Purkinje cell, opening voltage-sensitive calcium channels. The opening of the calcium channels allows intracellular calcium concentrations to rise momentarily, acting in concert with the input of the parallel fiber. This increase in intracellular calcium concentration has been shown to be necessary because the injection of bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA) into the Purkinje neuron prevents LTD, presumably by buffering the increase in intracellular calcium [15]. Kasono and Hirano [16] were able to show that the release of calcium inside the Purkinje cell could replace the climbing fiber stimulation, suggesting that the role of the climbing fiber in LTD is the depolarization and subsequent opening of calcium channels in the Purkinje cell membrane. However, Ca^{2+} influx alone is not sufficient to induce LTD in the Purkinje cell.

The role of the parallel fiber in the formation of LTD, though, has been debated much more. Lev-Ram *et al.* [17] have shown that the role of the parallel fiber is the production of NO which crosses the parallel fiber—Purkinje cell synaptic junction and is necessary for LTD induction. The NO released from the parallel fiber synapse acts on its target in the Purkinje cell. Experimentally, the release of NO in the Purkinje cell can mimic parallel fiber stimulation in LTD, suggesting that the role of the parallel fiber is the generation of NO as a messenger in the Purkinje cell [17]. To understand the timing and order of the pathways involved, Lev-Ram *et al.* [18] used caged messengers to study LTD. Caged messengers are biologically inert until they are activated by photolysis. When UV light is applied, the caged messengers are released and diffuse within the cell, simulating messenger release [19]. Caged NO and Ca^{2+}, when released simultaneously, can mimic the stimulation by climbing and parallel fibers, resulting in LTD. The NO released from the parallel fiber was shown to activate sGC in the cytoplasm of the Purkinje neuron (Fig. 2) [20]. Although releasing caged NO and Ca^{2+} could induce LTD, this effect could be blocked by inhibiting guanylyl cyclase with 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) or 6-anilino-5,8-quinolinedione (LY83583). This blockade can be reversed by adding caged cGMP, suggesting that, indeed, NO is activating sGC and that the cGMP is acting downstream of NO [18]. This indicates that the NO released from the parallel fibers acts to increase the activity of sGC in the Purkinje neuron, increasing the concentration of cGMP to further potentiate the signal. This increase in cGMP is still insufficient to induce LTD. Ca^{2+} elevation in the Purkinje cell is still required for LTD. The intracellular effector for Ca^{2+} in the Purkinje cell has not been identified; however, it is possible that in analogy with hippocampal LTD [21] calcineurin is involved. Also as yet undefined in this system is the identity of the coincident detector. In spite of these gaps in our current knowledge, it is clear that signals from both the parallel and climbing fibers are required to produce LTD in the Purkinje neuron, thus establishing the presence of a coincident detector in this cell. These pathways are summarized in Fig. 2.

GATING: PKA REGULATION OF THE RAS TO ERK 1,2 SIGNALING

It is now well established that many growth factors and mitogens stimulate proliferation through the activation of the GTP binding protein Ras that in turn activates the protein kinase Raf [22]. Raf then transmits the mitogenic signal through a kinase cascade to ERK-1 and -2 [23, 24]. In

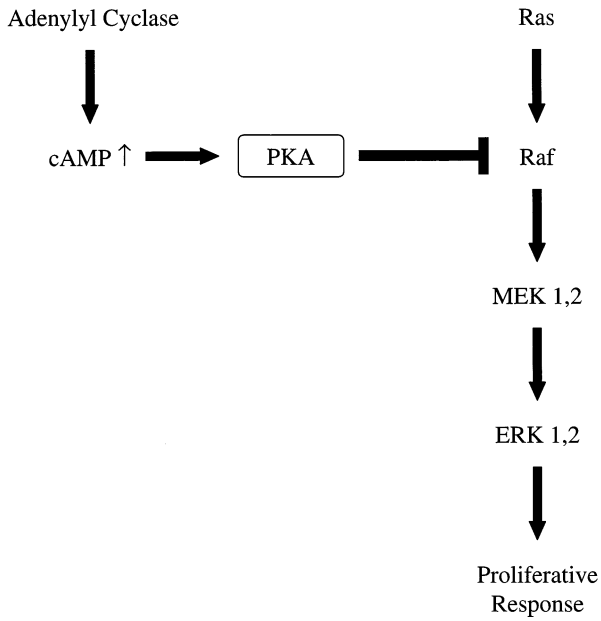

FIG. 3. Gating of the Ras to MAPK signaling pathway by cAMP. Stimulation of adenylyl cyclase results in an increase in cAMP, which can activate PKA. PKA can phosphorylate Raf kinase, resulting in a decrease in its catalytic activity. This inhibition of Raf activity leads to a decrease in the amount of activated MAPK in response to growth factor signaling and Ras activation, resulting in an inhibition of cell proliferation.

many cell types, activation of ERK1,2 is necessary, although not always sufficient for the triggering of proliferation. Analysis of this pathway had shed light on the mechanisms by which the cAMP pathway regulates proliferation. It has been known for over 25 years that elevation of cAMP blocks proliferation of certain cell types such as fibroblasts, T cells, and vascular smooth muscle cells [25]. Studies on how the cAMP pathway connects to the Raf-ERK1,2 pathway have shed light on the mechanism of inhibition (Fig. 3). Elevation of cAMP blocks Ras-dependent activation of ERK1,2 [26, 27] and Ras-induced transformation [11]. The locus of regulation is at the level of Raf. PKA phosphorylation of Raf has multiple effects. It prevents Raf association with Ras, thus preventing signal transfer. It also inhibits Raf catalytic activity since v-Raf-induced transformation is also blocked [28]. In this configuration, the cAMP pathway and PKA act solely as regulators, regulating signal flow from Ras to ERK-1,2. This phenomenon is termed gating, inasmuch as the regulatory pathway only "gates" signal flow through the transmittal pathway. A defining feature of gating interaction is the capability of one of the components of the transmittal pathway to be regulated (i.e. gated) by the gating pathway. This feature has been best exemplified by recent studies with Raf isoforms. Cells such as NIH-3T3 fibroblasts contain Raf-1 (c-Raf), which is negatively regulated by PKA. In contrast, PC-12 cells, neurons, and endocrine cells contain B-Raf, an isoform of Raf that is activated by PKA. The GTP binding protein Rap1, which is phosphorylated by PKA, mediates PKA activation of B-Raf. PKA phosphorylation increases GTP loading of Rap1, which in turn activates B-Raf, resulting in the activation of ERK-1,2 [29]. Thus, the molecular identity of Raf is a crucial determinant of whether cAMP gates signal transmittal to ERK1,2 or uses ERK1,2 to regulate transcription.

FEEDBACK: REGULATION OF LIGHT ADAPTATION IN THE RETINA

Rhodopsin is activated by light. Activated rhodopsin couples to transducin (G_t) inducing the α subunit, to bind GTP. The $\beta\gamma$ subunits then dissociate from the α subunit, enabling GTP-$G\alpha_t$ to activate phosphodiesterase, leading to a breakdown in cGMP. This decrease causes cGMP gated channels in the rod outer segment to close, decreasing the influx of Ca^{2+} and Na^+, resulting in the hyperpolarization of the membrane, which produces the neural impulse.

The mammalian eye is able to adapt to changes in lighting conditions through regulation of the rhodopsin-signaling pathway. When the eye has adapted to the ambient light, the amplitude and duration of the neural signal are altered. This adaptation to the level of illumination may proceed through a feedback mechanism in which rhodopsin signaling is modulated by phosducin, a protein that interacts with G-protein subunits. Unphosphorylated phosducin binds to $G\beta\gamma$, preventing it from reassociating with $G\alpha_t$, while phosducin that is phosphorylated by PKA has low affinity for $G\beta\gamma$ and, hence, does not affect trimer formation. By blocking the reassociation, the amount of heterotrimeric transducin available for activation by rhodopsin is decreased. This leads to a decrease in signal flow from rhodopsin to the cGMP phosphodiesterase.

The rhodopsin-signaling pathway is dependent on a series of proteins and messengers to convert the light signal into an electrical impulse (Fig. 4). The activation of phosphodiesterase causes the concentration of cGMP to fall, resulting in the closing of the cGMP-gated cation channel and, thus, a decrease in intracellular Ca^{2+} concentration. Willardson *et al.* [30] have shown that the fall in intracellular Ca^{2+} causes a decrease in the activity of an adenylyl cyclase through a Ca^{2+}/calmodulin-dependent mechanism. The decrease in activity of adenylyl cyclase along with the activation of phosphodiesterase leads to a decrease in cAMP levels. As cAMP levels fall, PKA activity is reduced. PKA phosphorylates phosducin, while a phosphatase dephosphorylates it. The phosphorylation state of phosducin dictates whether or not it can bind $G\beta\gamma$ [31–33], and, therefore, if phosducin can reduce signal flow from rhodopsin to the cGMP phosphodiesterase. Because phosducin is dephosphorylated upon light exposure and $G\beta\gamma$ is released from the trimer, phosducin is able to bind up the $\beta\gamma$ complex, thus reducing the concentration of the trimer, and, in effect, setting up a feedback inhibition of the rhodopsin pathway. Thus, the sensitivity of the system decreases. More light input is then required to activate the signaling pathway, which enables the retina to adapt to the high-light conditions. In a dark environment, the sensitiv-

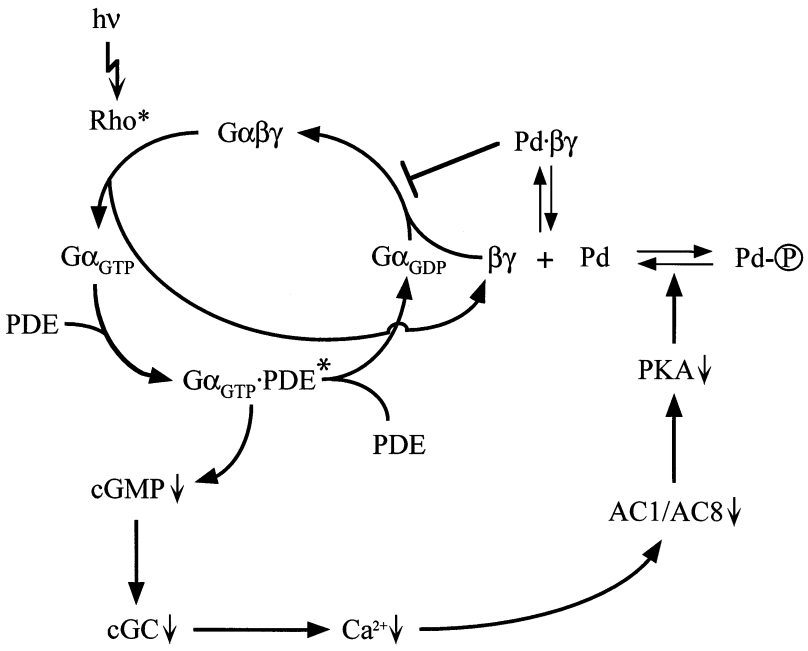

FIG. 4. Phosducin regulation of the rhodopsin signaling pathway in retinal light adaptation. Light-activated rhodopsin (Rho*) stimulates GTP binding to the heterotrimeric G-protein transducin. The Gα subunit binds GTP and dissociates from the Gβγ subunits. The α subunit activates cGMP-phosphodiesterase (PDE*), which breaks down cGMP. The fall in cGMP levels closes cGMP-gated calcium channels, leading to a decrease in intracellular Ca^{2+} concentration. This inactivates Ca^{2+}/calmodulin-sensitive adenylyl cyclases (AC1 and AC8), resulting in a decrease in cAMP. PKA activity drops, leading to the accumulation of unphosphorylated phosducin, which interacts with and sequesters Gβγ subunits. Because the βγ subunits are sequestered by phosducin, the amount of heterotrimeric transducin is reduced, resulting in decreased signal flow from rhodopsin to cGMP-PDE. This regulatory feature leads to light adaptation by adjusting the sensitivity of the signaling pathway.

ity of the signaling system is increased because phosducin is phosphorylated by the increased activity of PKA. The phosphorylated phosducin does not interact with Gβγ, therefore promoting trimer formation, which increases the amount of transducin available for activation. Thus, the original light signal through Ca^{2+} can modulate the cAMP pathway. This modulation results in feedback regulation of rhodopsin–transducin coupling and, therefore, enables this signaling pathway to regulate itself and adapt to the environmental conditions.

Since its discovery in the retina, phosducin has been found to be widely distributed and thought to regulate many G-protein signaling pathways such as G_o, G_i, and G_t [31, 32]. Receptor activation of G protein occurs through a conserved mechanism in all tissues. The Gα subunit binds GTP and dissociates from the Gβγ complex; both subunits are then free to associate with and regulate downstream effectors. The phosducin protein in its dephosphorylated state regulates the G-protein pathway by binding to and sequestering the Gβγ complex [31, 33]. The binding of phosducin to Gβγ occludes the Gα binding site on Gβ [34], thus preventing the reassociation of the heterotrimeric G protein with the receptor for further signaling and stimulation. The phosducin binding site on Gβ [34] also overlaps with the effector interaction regions of Gβ [35], thus allowing the phosducin signal to inhibit Gβγ signaling. PKA phosphorylates phosducin, preventing its association with Gβγ [31]. G_s-coupled receptors can control the levels of cAMP and, therefore, activation of PKA, which affects the phosphorylation state of phosducin. This suggests that the cAMP signaling pathway may feedback and regulate itself [31], as well as regulate other G-protein pathways, by using phosducin as a gate.

CONCLUSIONS

As individual signaling pathways become better characterized, our attention will be increasingly focused on how signaling pathways interact and the biological consequences of these interactions. It is becoming clear that in order to understand how signaling pathways interact, one needs to understand the molecular diversity of the signaling components that comprise the pathways and the functional consequences of this diversity. Thus, the molecular identity of components that are present in a certain pathway will determine how that pathway will interact with other pathways. It appears likely that higher orders of organization, resulting in networks that are composed of multiple signaling pathways, will be able to integrate and sort signals, both spatially and temporally, and thus exhibit the capability to acquire and exhibit "learned" behavior.

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