CHAPTER

4

Intracellular Signaling

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Almost all aspects of neuronal function, from its maturation during development, to its growth and survival, cytoskeletal organization, gene expression, neurotransmission, and use-dependent modulation, are dependent on intracellular signaling initiated at the cell surface. The response of neurons and glia to neurotransmitters, growth factors, and other signaling molecules is determined by their complement of expressed receptors and pathways that transduce and transmit these signals to intracellular compartments; and the enzymes, ion channels, and cytoskeletal proteins that ultimately mediate the effects of the neurotransmitters. The molecules involved in signal transmission and transduction are highly represented in mammalian and invertebrate genomes. Individual neuronal responses are further determined by the concentration and localization of signal transduction components, many of which can be modified by the prior history of neuronal activity. Several primary classes of signaling systems, operating at different time courses, provide great flexibility for intercellular communication. One class comprises ligand-gated ion channels, such as the nicotinic receptor considered in Chapter 10. This class of signaling provides fast transmission (see Chapter 16) that is activated and deactivated within 10 ms. It forms the underlying "hard wiring" of the nervous system that makes rapid multisynaptic computations possible. A second class consists of receptor tyrosine kinases, which typically respond to growth factors and to trophic factors and produce major changes in the growth, differentiation, or survival of neurons (Chapter 8). A third and largest class utilizes G protein-linked signals in a multistep process that slows the response from 100–300 ms to many minutes (see Chapters 10 and 16). The relatively slow speed is offset, however, by a richness in the diversity of its modulation and its inherent capacity for amplification and plasticity. The initial steps in this signaling system typically generate a second messenger inside the cell, and this second messenger then activates a number of proteins, including protein kinases that modify cellular processes. Signal transduction also modulates the level of transcription of genes, which determine the differentiated and functional state of cells (Chapter 5).

SIGNALING THROUGH G-PROTEIN-LINKED RECEPTORS

Signal transduction through G protein-linked receptors requires three membrane-bound components: (1) a cell surface receptor that determines to which signal the cell can respond; (2) a G protein on the intracellular side of the membrane that is stimulated by the activated receptor; and (3) either an effector enzyme that changes the level of a second messenger or an effector channel that changes ionic fluxes in the cell in response to the activated G protein. The human genome encodes for more than 800 receptors for catecholamines, odorants, neuropeptides, and light (see Chapter 10 for additional details of G protein coupled receptors) that couple to one or more of the 16 identified G proteins. These, in turn, regulate one or more of more than two dozen different effector channels and enzymes. The key feature of this information flow is the ability of G proteins to detect the presence of activated receptors and to amplify the signal by altering the activity of appropriate effector enzymes and channels.

G proteins are GTP-binding proteins that couple the activation of G-protein coupled receptors (GPCRs) by neurotransmitters at the cell surface to changes in the activity of effector enzymes and effector channels. A common effector enzyme is adenylyl cyclase, which synthesizes cyclic AMP (cAMP)—an intracellular

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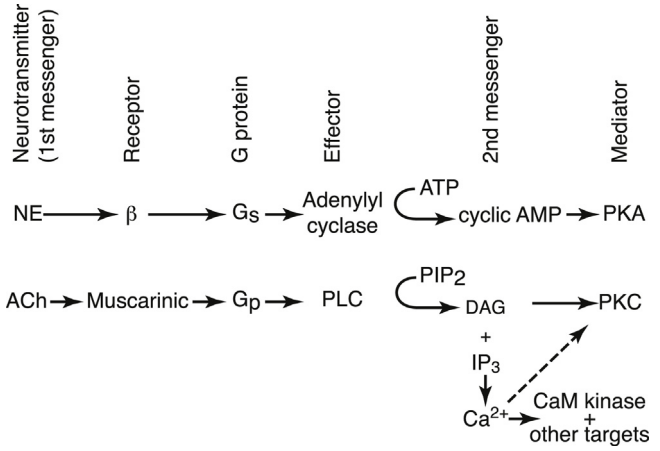

FIGURE 4.1 **Overview of G-protein signaling to protein kinases.** Norepinephrine (NE) and acetylcholine (ACh) can stimulate certain receptors that couple through distinct G proteins to different effectors, which results in increased synthesis of second messengers and activation of protein kinases (PKA and PKC). PLC, phospholipase C; PIP_2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; CaM, Ca^{2+}-calmodulin-dependent; IP_3, inositol 1,4,5-trisphosphate.

surrogate for the neurotransmitter, the first messenger. Phospholipase C (PLC), another effector enzyme, generates diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), the latter of which releases intracellular stores of Ca^{2+}. Information from an activated receptor flows to the second messengers that typically activate protein kinases, which modify a host of cellular functions. Cyclic AMP, Ca^{2+}, and DAG have in common the ability to activate protein kinases with broad substrate specificities. Protein kinases phosphorylate key intracellular proteins, ion channels, enzymes, and transcription factors taking part in diverse cellular biological processes. The phosphorylation state of these kinase targets is also determined by the activity of protein phosphatases and provides for a dynamic signal transduction process. In addition to regulating protein kinases, second messengers such as cAMP, cyclic GMP (cGMP), Ca^{2+}, and arachidonic acid can directly gate, or modulate, ion channels. G proteins can also couple directly to ion channels without the interception of second messengers or protein kinases. In these diverse ways, a neurotransmitter outside the cell can modulate essentially every aspect of cell physiology and encode the history of cell stimuli in the form of altered activity and expression of its cellular constituents. An overview of G-protein signaling to protein kinases is presented in Fig. 4.1.

G-Protein Signaling Operates on Common Principles

The many types of G proteins and the many types of effector enzymes have certain features in common (Hille, 1992). First, each receptor can couple to one or only a few G proteins, thus specifying the stimulus response. Second, the second messengers are typically synthesized in one or two steps from a precursor (e.g., ATP) that is itself inactive as a signaling molecule. G proteins can also stimulate enzymes that eliminate second messengers. Third, the initial signal can be greatly amplified: each receptor can activate many G protein molecules; each adenylyl cyclase can synthesize many cAMP molecules; and each protein kinase can phosphorylate many copies of each of its substrates. Fourth, the process is slower in onset and persists longer than ligand-gated ion channel signaling.

A fifth feature of G-protein signaling is the ability to orchestrate a variety of effects through the same second messenger. For example, the cAMP-dependent protein kinase (PKA), stimulated by serotonin exposure to sensory neurons in the mollusk *Aplysia*, rapidly phosphorylates membrane channels, which results in a greater influx of Ca^{2+} with each action potential in the sensory neuron and a subsequent short-term enhancement (short-term sensitization) of a gill-withdrawal response (see Chapters 18 and 20). On a slower time course, PKA also regulates the transcription of genes and translation of proteins that ultimately modify the number of synaptic contacts made by the sensory neuron. These effects are necessary to maintain the long-term memory for sensitization.

A nervous system with information flow by fast transmission alone would be capable of stereotyped or reflex responses. Integration and modulation of behavior is enabled by the diversity of signaling molecules with nearly unlimited flexibility of response over a broad time scale. This signaling is a key feature of neuronal plasticity, regulating every step of the way from neurotransmitter receptors and ion channels, signal transduction pathways, neurotransmitter synthesis, and release to the expression of genes in the nucleus that underlie synaptic changes linked to learning and memory (see Chapters 18 and 20 for extended discussion).

Receptors Catalyze the Conversion of G Proteins into the Active GTP-Bound State

G proteins undergo a molecular switch between two interconvertible states that are used to "turn on" or "turn off" downstream signaling. G proteins taking part in signal transduction utilize a regulatory motif that is seen in other GTPases engaged in protein synthesis and in intracellular vesicular traffic. G proteins are switched on by stimulated receptors, and they switch themselves off after a time delay. The G proteins are inactive when GDP is bound and are active when GTP is bound. The main function of GPCRs in

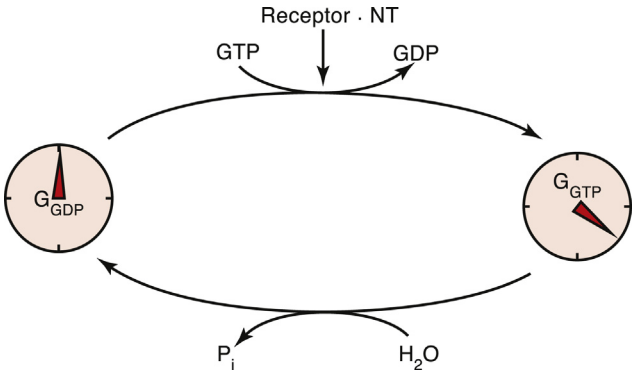

FIGURE 4.2 **GTPase activity of G proteins serves as a timer and amplifier.** Receptors activated by neurotransmitters (NT) initiate the GTPase timing mechanism of G proteins by displacement of GDP by GTP. Neurotransmitters thus convert G_{GDP} ("turned-off state") to G_{GTP} (time-limited "turned-on" state). At some point, the GTPase activity of the G-protein hydrolyzes GTP leading to return to the GDP bound (inactive) state.

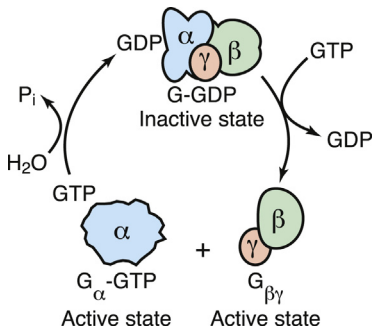

FIGURE 4.3 **Interconversion, catalyzed by excited receptors, of G-protein subunits between inactive and active states.** Displacement of GDP with GTP dissociates the inactive heterotrimeric G protein, generating α-GTP and βγ, both of which can interact with their respective effectors and activate or inhibit them. The system converts into the inactive state after GTP has been hydrolyzed and the subunits have reassociated. *From* Biochemistry, *4th ed. by Stryer.* © *1995 by Lubert Stryer. Used with permission of W. H. Freeman and Company.*

activating G proteins is to catalyze an exchange of GTP for GDP. This is a temporary switch because G proteins have an inherent GTPase activity that hydrolyzes the bound GTP and converts the G protein back into the GDP-bound, or inactive, state. Thus, a G protein must continuously sample the state of activation of the receptor, and it transmits downstream information only while the neuron or glial cell is exposed to neurotransmitter. A fast GTPase means that the signal transduction pathway is very responsive to the presence of neurotransmitter outside and can respond to individual stimuli even at high frequency. A slow GTPase provides greater amplification but cannot produce distinct responses to each stimulus presented at high frequency because of its long latency for returning to the basal state. The GTPase activity of G proteins serves both as a timer and as an amplifier (Fig. 4.2). The Nobel Prize was awarded in 1994 to Alfred Gilman and Martin Rodbell for the fundamental discovery of G-proteins and their role in signal transduction and the Nobel Prize in 2012 was awarded to Robert Lefkowitz and Brian Kobilka for the identification and purification of the β-adrenergic receptor (a prototypical GPCR) and elucidation of its structure that couples to G-proteins.

The G-Protein Cycle

G proteins are trimeric structures composed of two functional units: (1) an α subunit (39−52 kDa) that catalyzes the GTPase activity and (2) a βγ dimer (35 and 8 kDa, respectively) that tightly interacts with the α subunit when bound to GDP (Stryer and Bourne, 1986; Neer, 1995; Birnbaumer, 2007). The role of the three subunits in the G-protein cycle is depicted in Fig. 4.3 and 4.4. In the basal state, GDP is bound tightly to the α subunit, whose interaction with its effectors is blocked by association with the βγ pair to form an inactive G protein. Binding of the neurotransmitter to the receptor produces a conformational change that positions previously buried residues that promote increased affinity of the receptors for the inactive G protein (Palczewski et al., 2000). Coupling with the activated receptor reduces the affinity of the α subunit for GDP, facilitating its dissociation and thus leaving the nucleotide binding site empty. Either GDP or GTP can bind to the vacant site; however, because the level of GTP in the cell is much greater than the level of GDP, the dissociation of GDP is usually followed by the binding of GTP. Thus, the stimulated receptor effectively catalyzes an exchange of GTP for GDP (Cassel and Selinger, 1978). A given receptor can interact with only one or a limited number of G proteins, and the α subunit generates most of this specificity. Binding of GTP has two consequences: (1) it dissociates the G protein into α-GTP and βγ, and (2) the α-GTP subunit has a reduced affinity for the stimulated receptor, leading to dissociation of the complex (Sprang et al., 2007).

The GDP−GTP exchange is inherently very slow, on the order of many minutes, and ensures that very little of the G protein is in the on state under basal conditions. The level of G protein in the "on" state can increase from 1% to more than 50% of all G protein through stimulated receptor interactions. The direction of the cycle is determined by the GTPase reaction, which uses the energy of GTP to make the reaction irreversible and to maintain a low level of α-GTP in the basal state (Stryer and Bourne, 1986).

Information Flow

One of the more tense and public debates in signal transduction has been the question of whether the α subunit alone conveys information that specifies

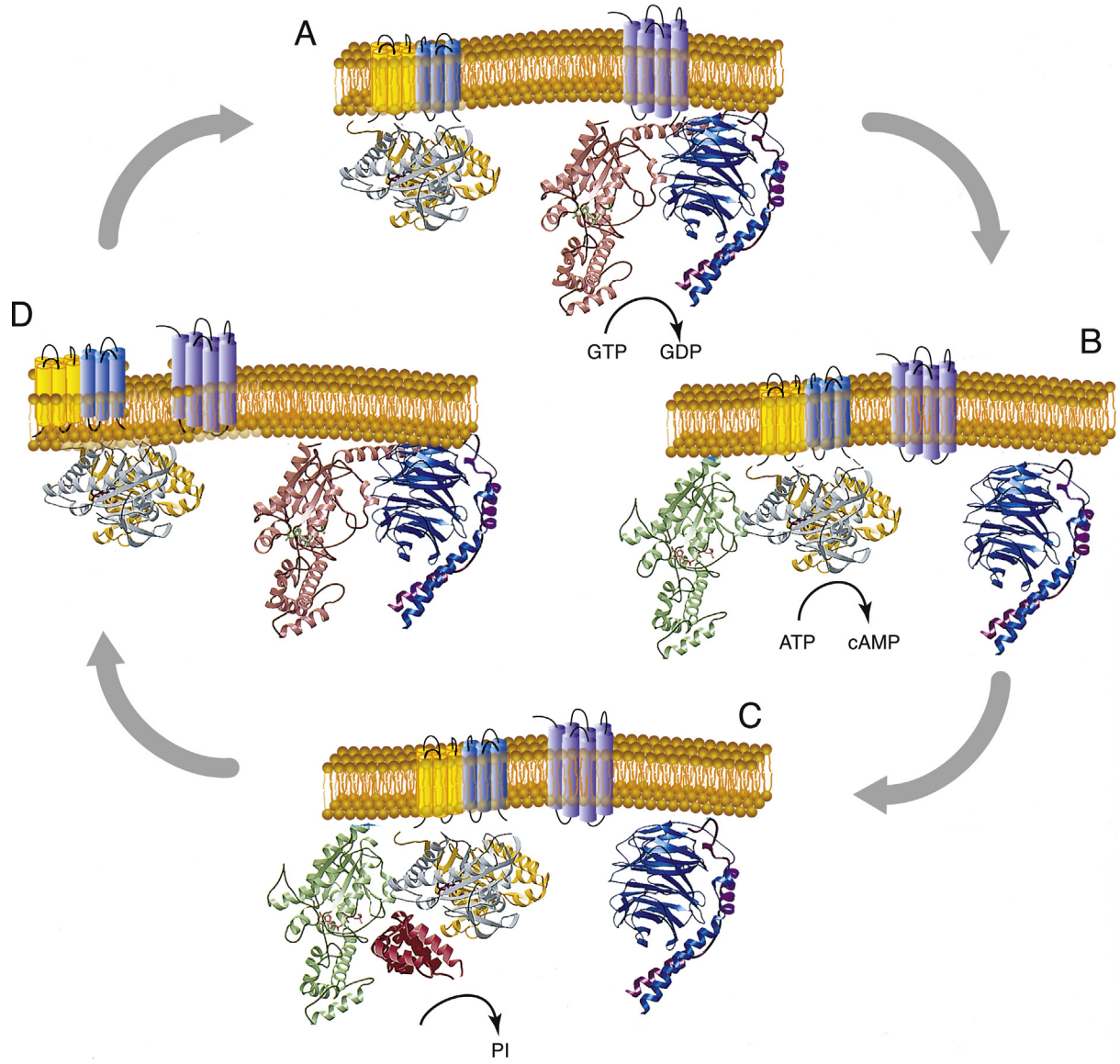

FIGURE 4.4 (A) G proteins are held in an inactive state because of very high affinity binding of GDP to their α subunits. When activated by an agonist, GPCRs (right, glowing magenta) interact with heterotrimeric G proteins (α, pink; β, blue; γ, burgundy illustrated as ribbon diagrams) and stimulate dissociation of GDP. This permits GTP to bind to and activate α, which then dissociates from the high-affinity dimer of β and γ subunits. (B) Both activated (GTP-bound) α (now shown in its active form, lime) and βγ are capable of interacting with downstream effectors. Also illustrated is the interaction of α_s-GTP with adenylyl cyclase (catalytic domains are mustard and ash). Adenylyl cyclase then catalyzes the synthesis of the second messenger cyclic AMP (cAMP) from ATP. (C) Signaling is terminated when α hydrolyzes its bound GTP to GDP. In some signaling systems, GTP hydrolysis is stimulated by GTPase-activating proteins or GAPs (cranberry) that bind to α and stablize the transition state for GTP hydrolysis. (D) Hydrolysis of GTP permits α-GDP to dissociate from its effector and associate again with βγ. The heterotrimeric G protein is then ready for another signaling cycle if an activated receptor is present. *The figure is based on the original work of Mark Wall and John Tesmer.*

which effector is activated or whether the βγ pair has a role. One of the contestants even paid for a vanity license plate proclaiming "α not β." The α subunit was thought to be responsible for specifying which effector enzyme was activated by a G protein (Cassel and Selinger, 1978). This notion was eventually changed when it was found that βγ can directly activate certain K^+ channels (Logothetis et al., 1987; Huang et al., 1995). The historic association of G-protein function with α has persisted for the purpose of nomenclature, with G_s and α_s referring to the G protein and its corresponding α subunit, which stimulates adenylyl cyclase. These names have been retained even though it is now apparent that α and βγ subunits can both modify effector enzymes and channels and that a given α subunit may combine with a number of βγ

pairs. The α subunits may act either independently or in concert with βγ (Clapham and Neer, 1993). Furthermore, β and γ subunits in a βγ pair can combine in many different ways. Other legacy terms include G_i, G_p, and G_o used for G-protein activities that inhibited adenylyl cyclase, stimulated phospholipase, or were presumed to have other effects, respectively. At this stage, cloning and genomic sequencing have outpaced functional studies. The inherent affinities of βγ pairs for a particular α and spatial segregation of G proteins probably greatly limit the number of combinations of subunits. See Box 4.1.

Function of α Subunits

Determination of the crystal structures of different conformational states of some α subunits and βγ subunits has been a source of insight into their functional domains and the critical GTPase activity of all α subunits (Sondek et al., 1996; Wall et al., 1996). The α subunits are compact molecules that must accommodate a number of protein–protein interactions in addition to their GTP-hydrolyzing activity. The β subunit consists of repeating motifs arranged like blades of a propeller or wedges of a pie with a central tunnel (see

BOX 4.1

WHY ARE G PROTEIN-REGULATED SYSTEMS SO COMPLEX?

Transmembrane signaling systems all contain two fundamental elements: one that recognizes an extracellular signal (a receptor) and another that generates an intracellular signal. These elements can be easily incorporated into a single molecule, for example, in receptor tyrosine kinases and guanylyl cyclases. Why then are G protein-regulated systems so complex, minimally containing five gene products in the basic module (receptor, heterotrimeric G protein, and effector)? The design of these systems permits both integration and branching at its two interfaces: between receptor and G protein and between G protein and effector. Each component of the system can thus be regulated independently—transcriptionally, posttranslationally, or by interactions with other regulatory proteins. Furthermore, hundreds of genes encode receptors for hormones and neurotransmitters, dozens of genes encode G-protein subunits (α, β, and γ), and dozens more genes encode G protein-regulated effectors. Each cell in an organism thus has the opportunity to sample the genome and construct a highly customized and sophisticated switchboard in its plasma membrane, permitting the organism to make an extraordinary variety of responses to complex situations. The choices that each cell makes include much more than just the components of the basic modules, extending as well to regulators of G protein-mediated pathways such as receptor kinases and GTPase-activating proteins. And, of course, the identity and concentrations of the components of the switchboard can be sculpted within minutes or hours to permit adaptation to developmental needs or environmental stresses.

The classic stress response of mammals to the hormone epinephrine provides an elegant example of the power of modular signal transduction systems. A single hormone is used to initiate responses of opposite polarity in very similar cells. Thus, vascular smooth muscle in skin contracts to minimize bleeding (if there is a wound) and to maintain blood pressure, whereas vascular smooth muscle in skeletal muscle relaxes to provide increased blood flow during heightened physical activity. Smooth muscle in the intestine relaxes, whereas cardiac muscle is powerfully stimulated. In addition, hepatocytes hydrolyze glycogen to glucose, adipocytes hydrolyze triglycerides and release free fatty acids for fuel, and certain endocrine and exocrine secretions are stimulated or inhibited. Several distinct receptors for epinephrine are selectively expressed in various cell types to achieve this beneficial orchestration of stimulatory and inhibitory events. These receptors vary in their capacities to interact with G proteins from three different subfamilies (G_s, G_i, G_q) and thus to activate or inhibit several effects to achieve the desired responses. Each cell's choices of particular receptors, G proteins, and effectors permit additional choices of regulators of each of these components to adjust the magnitude and/or the kinetics of the response.

The past two decades of research in this area have witnessed identification and characterization of the molecular players involved in G protein-mediated signaling and appreciation of the basic mechanisms that underlie the protein–protein interactions that drive these systems. Current research is expanding this basic core of knowledge: on the one hand, toward greater understanding of how the individual modules contribute to the integrated networks of intact cells and, on the other, toward elucidation of the physical and structural bases of these complex cellular reactions. We can begin to construct a movie of G protein-mediated signaling at atomic resolution, and many of its most important frames are shown in Fig. 4.4.

Alfred G. Gilman and Stephen R. Sprang

next subsection). In the trimeric GDP-bound state, the α subunit is docked to the β subunit (Wall et al., 1996). Receptors interact with the carboxy terminus of the α subunit; this interaction, along with other contacts, specifies which receptors can activate which α subunits (Conklin et al., 1993). The guanine nucleotide binding site is altered by interaction with ligand-bound receptors to facilitate dissociation of GDP and exchange for GTP, thus producing an activated α-GTP conformation. The γ phosphate present in GTP, but not in GDP, interacts with a region of α that participates in the docking of α to βγ. The resulting conformational changes reduce the affinity between this surface and βγ, resulting in dissociation of the trimer to produce α-GTP and βγ with exposed surfaces capable of interacting with their respective effectors. Dissociation also releases the receptor, which, if remaining in the activated state, can then catalyze activation of other G proteins. GTP hydrolysis reverses the conformational changes and enables a region of α to interact with the β subunit. The functions of α include both stimulation and inhibition of adenylyl cyclases that is sensitive to cholera toxin and pertussis toxin, respectively. In addition, it modulates activation of cGMP phosphodiesterase, PLC, and regulation of Na^+/K^+ exchange, PI3K, RhoGEF and rasGAP (Clapham and Neer, 1993; Birnbaumer, 2007).

Function of βγ Subunits

The crystal structure of the βγ heterodimer both in its inactive form bound to the α subunit and in its free active form has been determined (Sondek et al., 1996; Wall et al., 1996). The C-terminal half of the β subunit has a sevenfold repeat of a structural motif termed the WD repeat, which interacts with γ and is the likely site of interaction between β and its effector enzymes and channels. The WD repeat is a sequence of 25–40 amino acids and ends with the amino acids tryptophan (W) and aspartic acid (D). Each repeat forms a wedge of a circular disk with a central tunnel in both the free and bound states. The γ subunit is in an extended conformation, circling and making contact with several WD domains of the β subunit. Prenylation of the C terminus of γ anchors the βγ dimer to membranes.

The effector functions of βγ dimers include inhibition of many adenylyl cyclases and stimulation of adenylyl cyclase types II and IV (along with α). In addition, they regulate stimulation of phospholipase Cβ, K^+ and Ca^{2+} channels, phospholipase A_2, phosphatidylinositol-3-kinase, PKD and dynamin in vesicle budding. The identified effector targets of βγ are several adenylyl cyclases, the β isoform of phospholipase C, ion channels (for K^+ and Ca^{2+}), phospholipase A2, and phosphatidylinositol 3-kinase (Clapham and Neer, 1993; Birnbaumer, 2007). The other functions of βγ are as follows. First, they keep G proteins inactive in the basal state by complexing with α and reducing its intrinsic GTP–GDP exchange rate. Second, they help to target the α subunits to the membrane and increase the affinity of α-GDP for ligand-bound receptors. Third, both β and γ help to specify which receptors couple to the G protein. Fourth, the WD repeats of β serve as anchors for a protein kinase, called βARK, that terminates signaling by ligand-bound β-adrenergic receptors.

Examination and Manipulation of G-Protein-Coupled Signals

Neurotransmitters can produce their cellular effects by a variety of signal transduction pathways. A number of experimental tools and approaches have been applied to identify the specific G-proteins involved. These include use of antibodies to the G-protein, suppression of RNA or gene disruption such as knockouts, and reconstitution. In addition, G-proteins can be delineated by use of toxins and guanine nucleotides.

Toxins

Differential sensitivities to cholera toxin and pertussis toxin can be used to implicate a G-protein-mediated pathway. Both G_s and transducin are sensitive to cholera toxin, which selectively ADP-ribosylates α_s and α_t. The α subunits of G_i, G_o, and transducin, but not of G_s, are ADP-ribosylated by pertussis toxin. These toxins transfer the ADP-ribose moiety of NAD^+ to an arginine (cholera toxin) or cysteine (pertussis toxin) on the appropriate α subunit. The toxins act at distinct steps in the GTPase cycle to lock it in either the on or the off position (see Fig. 4.3). Cholera toxin inhibits the GTPase activity, thereby generating a persistently on state. For example, cholera toxin treatment of G_s stimulates robust and continuous production of cAMP by adenylyl cyclase. In contrast, pertussis toxin acts on the inactive G protein, blocking its interaction with receptors so that it cannot be activated and thus remains in the GDP-bound, or off, state. Toxin sensitivity can be used to narrow the choice of possible G proteins taking part in a process or to block a known pathway.

Guanine Nucleotides and NaF

The GTPase cycle of all G proteins can also be modified for experimental purposes by GTPγS or GDPβS. GTPγS is a nonhydrolyzable analog of GTP with high affinity for the α subunit. Activation of any of the G proteins in the presence of GTPγS leads to the

exchange of GTPγS for GDP to produce α-GTPγS. The G protein is thereby activated for prolonged periods because the GTPase cycle is blocked and remains so until GDP exchanges with GTPγS. A similar response is obtained by addition of aluminum fluoride. Fluoride forms a complex with trace amounts of Al^{3+} and binds to α-GDP. The aluminum fluoride moiety simulates the γ phosphate of GTP so that, like GTPγS, α-GDP-AlF_3 persistently activates the G protein. (Coleman et al., 1994; Sondek et al., 1994). In contrast, GDPβS can exchange with GDP, leaving the G protein in the inactive state.

Effector Enzymes, Channels, and Transporters Decode Receptor-Mediated Cell Stimulation in the Cell Interior

The function of the trimeric G proteins is to decode information about the concentration of neurotransmitters bound to appropriate receptors on the cell surface and convert this information into a change in the activity of enzymes and ion channels that mediate the effects of the neurotransmitter. The effector can be an enzyme that synthesizes or degrades a diffusible second messenger, a phospholipase, a transporter, or an increasing number of other effectors being identified.

Response Specificity in G-Protein Signaling

The modular design of G-protein signaling may appear to be incapable of providing specificity. Receptors can stimulate one or more G proteins, G proteins can couple to one or more effector enzymes or channels, and the resulting second messengers will affect many cellular processes. Signals originating from activated receptors can either converge or diverge, depending on the receptor and on the complement of G proteins and effectors in a given neuron (Ross, 1989).

How can a neurotransmitter produce a specific response if G-protein coupling has the potential for such a diversity of effectors? The specificity is partly determined by the restricted complement of receptors, G proteins, and effectors, expressed in a given neuron. Transducin, for example, is confined to the visual system, where the predominant effector is the cGMP phosphodiesterase and not adenylyl cyclase. A number of other factors combine to increase signal specificity (Hille, 1992; Neer, 1995; Sondek et al., 1994).

Specificity and choice. Receptors and G proteins have higher intrinsic affinities and efficacies for modulating the activity of the "correct" G protein(s) and effector (s), respectively.

Spatial compartmentalization. Second messenger systems can be compartmentalized, thus adding specificity and localized control of signaling. The same receptor may regulate a Ca^{2+} channel through one G protein at a nerve terminal and regulate PLCβ at a distal dendrite through another G protein. Addition of the neurotransmitter in culture would produce both effects, but synaptic inputs would be able to elicit distinct localized effects, i.e., regulation of the Ca^{2+} channel at the terminal and PLCβ at the dendrite.

GTPase activity. The degree of amplification by the G protein (based on GTPase) and by the effector (based on its specific activity or conductance) can determine which of the possible pathways is more prominent. Furthermore, some effectors appear to act as GTPase-activating proteins (GAPs), which modify the intrinsic GTPase activity of the G protein. Such an effector terminates signaling faster when stimulated by one G protein relative to another and fine-tunes the flow of information through the various forks of the signaling system. The signaling strength (i.e., the speed and efficiency) of any branch of the pathway can be modulated. Inherent affinities, level of expression of the various components, compartmentalization, GTPase rates, and GAP activity combine to produce either a well-focused response by a single pathway or a richer and more diffuse response through several pathways.

Fine-tuning of cAMP by Adenylyl Cyclases

The level of cAMP is highly regulated owing to a balance between synthesis by adenylyl cyclases and degradation by cAMP phosphodiesterases (PDEs). Each of these enzymes can be independently regulated and manipulated. Adenylyl cyclase was the first G-protein effector to be identified, and now a group of related adenylyl cyclases are known to be differentially regulated by both α and βγ subunits. (Taussig and Gilman, 1995). G proteins can both activate and inhibit adenylyl cyclases either synergistically or antagonistically.

Adenylyl cyclases are large proteins of approximately 120 kDa. They consist of a tandem repeat of the same structural motif—a short cytoplasmic region followed by six putative transmembrane segments and then a highly conserved catalytic domain of approximately 35 kDa on the cytoplasmic side (Krupinski et al., 1989). Some isoforms are activated by Ca^{2+} via calmodulin, a Ca^{2+}-binding protein and have one calmodulin binding domain in the link following the first catalytic domain (Halls and Cooper, 2011).

Differential regulation of adenylyl cyclase isoforms. All adenylyl cyclase isoforms are stimulated by G_s through its α_s subunit. Mammals have at least nine adenylyl cyclase isoforms, designated I–IX (not including alternative splicing), that differ in their regulatory properties and tissue distribution (Taussig and Gilman, 1995; Hanoune and Defer, 2001; Tang and

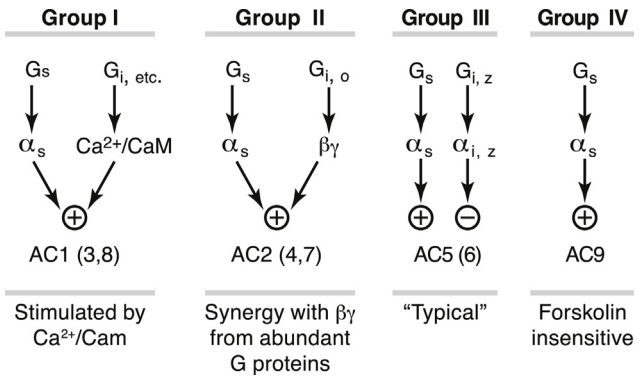

FIGURE 4.5 **Isoforms of adenylyl cyclase (AC).** All isoforms are stimulated by α_s but differ in the degree of interaction with Ca^{2+}-calmodulin and with $\beta\gamma$ derived from inhibitory G proteins. The four classes are shown as Group I to IV and the corresponding adenylyl cyclases, e.g., adenylate cyclase type 1 (also type 3 and 8) are shown below. Not shown is the ability of excess $\beta\gamma$ to complex with and inhibit group A and group C adenylyl cyclases. *Updated from Taussig and Gilman (1995).*

Gilman, 1991). They can be divided into four groups on the basis of additional regulatory properties (Fig. 4.5). Group I (AC1, 3, 8) is activated by Ca^{2+}-calmodulin. Group II (AC2, 4, 7) is weakly responsive to direct interaction with α_s or $\beta\gamma$ but is highly activated when both are present and can be activated by PKC (Krupinski et al., 1989; Halls and Cooper, 2011). As described later, the synergistic effect of α_s and $\beta\gamma$ enables this cyclase to function as a coincidence detector (see also Chapter 18). Group III (AC5, 6) is negatively regulated by inhibitory G proteins and Group IV (AC9) is a forskolin-insensitive cyclase.

Inhibition of adenylyl cyclases. Adenylyl cyclases are also subject to several forms of inhibitory control. First, activation of all adenylyl cyclases can be antagonized to some extent by $\beta\gamma$ released from abundant G proteins, such as G_i, G_o, and G_z, which complex with α_s-GTP and shift the equilibrium toward an inactive trimer by mass action. Second, either α or $\beta\gamma$ subunits derived from G_i, G_o, or G_z can directly inhibit group I cyclases, and the α subunit from G_i or G_z can inhibit Group III cyclases (Tang and Gilman, 1991). Many G proteins can generate $\beta\gamma$ subunits capable of directly inhibiting group A and activating group B adenylyl cyclases. However, not all G proteins are sufficiently abundant to produce enough $\beta\gamma$ to bring about these effects. The level of G_s in particular is low; thus, the α_s derived from G_s is sufficient to activate adenylyl cyclases, but the $\beta\gamma$ derived from it is insufficient to directly inhibit or activate adenylyl cyclases. Therefore, the sources of $\beta\gamma$ for modulation of Type I and II adenylyl cyclases are likely the abundant G proteins, such as G_i and G_o. This explains the apparent paradox that receptors that couple to G_s produce effects only through α_s, whereas receptors that couple to G_i produce effects through both α_i and $\beta\gamma$ even though they can share the same $\beta\gamma$.

Receptor coupling to adenylyl cyclase. Dozens of neurotransmitters and neuropeptides work through cAMP as a second messenger and do so by activation of either G_s to stimulate adenylyl cyclase or G_i or G_o to inhibit adenylyl cyclase being activated by stimulatory inputs. Among the neurotransmitters that increase cAMP are the amines norepinephrine, epinephrine, dopamine, serotonin, and histamine and the neuropeptides vasointestinal peptide (VIP) and somatostatin. In the olfactory system, a special form of G-protein α subunit, termed α_{olf}, serves the same function as α_s. Odorants are detected by hundreds of GPCRs that activate G_{olf}, which in turn activates adenylyl cyclase AC3 in the neuroepithelium. Many of the same neurotransmitters activate distinct receptors that couple to G_i or G_o. They include acetylcholine, dopamine, serotonin, norepinephrine, and opiate peptides.

Adenylyl cyclases as coincidence detectors. Adenylyl cyclases can integrate synaptic input and even detect concurrent stimulation of neurons by two or more neurotransmitters (Bourne and Nicoll, 1993). AC1, for example, is stimulated by neurotransmitters that couple to G_s and by neurotransmitters that elevate intracellular Ca^{2+}. This adenylyl cyclase can convert the depolarization of neurons and associated Ca^{2+} elevation into an increase in cAMP (Wayman et al., 1994), a likely basis for its role in several associative forms of learning (Levin et al., 1992; Ocorr et al., 1985; Wu et al., 1995; Yovell and Abrams 1992).

Stimulation of AC2 adenylyl cyclase by α_s is conditional on the presence of $\beta\gamma$ derived from a G protein other than G_s, thus enabling the cyclase to serve as a coincidence detector (Tang and Gilman, 1991; Federman et al., 1992). As indicated earlier, $\beta\gamma$ derived from G_s is not sufficient to produce synergistic activation of this enzyme. Thus, activation of a second receptor, presumably coupled to the abundant G_i and G_o, is needed to provide the $\beta\gamma$. In cortex and hippocampus, which contain the type II adenylyl cyclase, neurotransmitters coupling to G_i can potentiate increases in cAMP resulting from concurrent stimulation by neurotransmitters coupled to G_s.

Sources of Second Messengers: Phospholipids

Two phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylcholine (PC), are primary precursors for a G protein-based second messenger system. Three second messengers—diacylglycerol, arachidonic acid and its metabolites, and elevated Ca^{2+}—are ultimately produced. A single step converts the inert phospholipid precursors into the lipid messengers (Divecha and Irvine, 1995). Ca^{2+}

becomes functionally active by its regulated entry into the lumen of the cytosol or nucleus from a sequestered pool in the endoplasmic reticulum or extracellular space. DAG action is primarily mediated by protein kinase C (Takai et al., 1979; Tanaka and Nishizuka, 1994). Ca^{2+} has many cellular targets but mediates most of its effects through Ca^{2+}-calmodulin which in turn regulates multiple enzymes and processes.

Generation of DAG and IP_3 from G_q and G_i coupled to PLCβ. The phosphatidyl inositide-signaling pathway is just as prominent in neuronal signaling as the cAMP pathway and is similar to it in overall design (Berridge, 2009). Stimulation of a large number of neurotransmitters and hormones [including acetylcholine (M1, M3), serotonin ($5HT_2$, $5HT_{1C}$), norepinephrine (α_{1A}, α_{1B}), glutamate (mGluR), neurotensin, neuropeptide Y, and substance P] is coupled to the activation of a phosphatidylinositide-specific PLC. In recent years, it has become clear that in addition to their function as membrane phospholipids, the phosphoinositides are precursors for second messengers (Berridge, 2009; Di Paolo and De Camilli, 2006).

Phosphatidylinositol (PI) is composed of a DAG backbone with *myo*inositol attached to the *sn*−3 hydroxyl by a phosphodiester bond (Fig. 4.6). The six positions of the inositol are not equivalent: the 1-position is attached by a phosphate to the DAG moiety. PI is phosphorylated by PI kinases at the 4-position and then at the 5-position to form PIP_2. In response to the appropriate G-protein coupling, PLC hydrolyzes the bond between the *sn*−3 hydroxyl of the glycerol backbone and the phosphoinositol to produce two second messengers—DAG, a hydrophobic molecule, and inositol 1,4,5-trisphosphate (IP_3), which is water soluble (Divecha and Irvine, 1995; Hokin and Hokin, 1955) (Fig. 4.6). There are six classes of PLCs

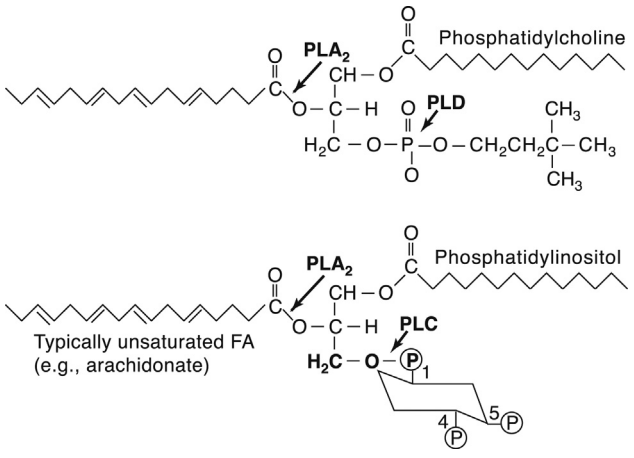

FIGURE 4.6 **Structures of phosphatidylinositol and phosphatidylcholine.** The sites of hydrolytic cleavage by PLC, PLD, and PLA_2 are indicated by arrows. FA, fatty acid.

that hydrolyze PIP_2 (PLCβ, γ, δ, ε, ζ, and η) with a common catalytic domain structure but different regulatory properties. G proteins couple to several variants of PLCβ. PLCγ is regulated by growth factor tyrosine kinases. Less is known about regulation of the others, but PKCε can be regulated by both G proteins and small GTPases.

PLCβ is coupled to neurotransmitters by G_q and G_i. A pertussis toxin-sensitive pathway is mediated by a number of isoforms originally termed G_q and mediated by their α_q subunit. G_i is coupled to PLCβ via its βγ rather than α subunit in a pathway that is insensitive to pertussis toxin. Receptor tyrosine kinases can regulate PLCγ by a G-protein-independent pathway involving their recruitment to the receptor and activation via phosphorylation.

DAG is derived from activation of phospholipase D. A slower but larger increase in DAG can be generated by activation phospholipase D (PLD) which cleaves phosphatidylcholine to produce phosphatidic acid and choline. Dephosphorylation of phosphatidic acid produces DAG. The PLD pathway may be used by some mitogens and growth factors and likely contains a variety of activation schemes that may include G proteins. (Divecha and Irvine, 1995; Nishizuka, 1995).

Regulation of PLCγ by receptor tyrosine kinases. DAG and IP_3 are also produced when certain receptor tyrosine kinases are activated and are independent of G proteins. Stimulation of receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor leads to their autophosphorylation on tyrosine residues and activation. Specific phosphotyrosine moieties on the receptor then recruit effector enzymes, such as PLCγ, that possess Src homology 2 (SH2) domains. These structural elements specifically recognize certain protein sequences with phosphotyrosine and lead to the translocation of effectors such as PLCγ to the receptor at the membrane. After binding to the receptor, PLCγ is activated by phosphorylation on tyrosine and hydrolyzes PIP_2 to DAG and IP_3.

Additional lipid messengers. DAG is itself a source of another lipid messenger generated by the action of phospholipase A_2 (PLA_2) which releases the fatty acid, typically arachidonic acid, from the sn−2 position of the DAG backbone (Fig. 4.6). PLA_2 can be activated by βγ and α subunits of G proteins. A cytosolic PLA_2 may mediate growth factor signaling via mitogen-activated protein (MAP) kinases. Phosphorylation of this PLA_2 leads to its translocation to the membrane, where it can act on membrane phospholipids. Arachidonic acid has biological activity of its own in addition to serving as a precursor for prostaglandins and leukotrienes. Arachidonic acid and other *cis*-unsaturated fatty acids can modulate K^+ channels, PLCγ, and some forms of PKC.

The phosphatidylinositides exist with modifications at the 3, 4, and 5 position to generate a family of seven lipids, [PI3P, PI4P, PI5P, PI(3,4)P2, PI(4,5)P2, PI(3,5)P2, and PI(3,4,5)P3] that mediate important structural or enzymatic effects. They bind to specific protein binding domains to regulate vesicular traffic and/or protein kinases involved in survival and cell death. Mutations in their synthesis and metabolism have pathophysiological consequences (McCrea and De Camilli, 2009). There is evidence that another lipid, sphingomyelin, is a precursor for intracellular signals as well.

IP_3, a potent second messenger that produces its effects by mobilizing intracellular Ca^{2+}. The main function of IP_3 is to stimulate the release of Ca^{2+} from intracellular stores (Streb et al., 1983). The concentration of cytosolic free Ca^{2+} is approximately 100 nM in unstimulated neurons, whereas its concentration in the extracellular space is 1.5–2.0 mM. This provides a tremendous driving force for Ca^{2+} movement down its concentration gradient; its reversal potential is more than 100 mV. Ca^{2+} is the most common second messenger in neurons, yet it can be neurotoxic. Neurons have therefore developed several mechanisms for maintaining a low interstimulus level of free Ca^{2+}. A Ca^{2+}-ATPase and a Na^{+}–Ca^{2+} exchanger in the plasma membrane catalyze the active transport of Ca^{2+} to the extracellular space, and a different Ca^{2+}-ATPase in the ER membrane sequesters Ca^{2+} in the ER network. Much of the Ca^{2+} in the ER is complexed with low-affinity-binding proteins that enable the ER to concentrate Ca^{2+} yet enable Ca^{2+} to readily flow down its concentration gradient into the cell lumen on opening of Ca^{2+} channels in the ER. The ER is the major IP_3-sensitive Ca^{2+} store in cells (Fig. 4.7).

The IP_3 receptor is a macromolecular complex that functions as an IP_3 sensor and a Ca^{2+} release channel. It has a broad tissue distribution but is highly concentrated in the cerebellum. The IP_3 receptor is a tetramer of 313-kDa subunits with a single IP_3-binding site at the N-terminal region of each subunit, facing the cytoplasm. Ca^{2+} release by IP_3 is highly cooperative so that a small change in IP_3 has a large effect on Ca^{2+} release from the ER. The IP_3 receptor has low activity at either high or low levels of cytoplasmic Ca^{2+}, with peak release requiring 200–300 nM Ca^{2+}, a property that may be used in the generation of some Ca^{2+} waves (Berridge, 1993; Tsien and Tsien, 1990; Bootman et al., 1997). The mouse mutants *pcd* and *nervous* have deficient levels of the IP_3 receptor and exhibit defective Ca^{2+} signaling, and a genetic knockout of the IP_3 receptor leads to motor and other deficits.

Termination of the IP_3 signal. IP_3 is a transient signal terminated by dephosphorylation to inositol. Inactivation is initiated either by dephosphorylation to inositol 1,4-bisphosphate (Fig. 4.7) or by an initial phosphorylation to a tetrakisphosphate form that is dephosphorylated by a different pathway. Both pathways have in common an enzyme that cleaves the phosphate on the 1-position. Complete dephosphorylation yields inositol, which is recycled in the biosynthetic pathway. Recycling is important because most

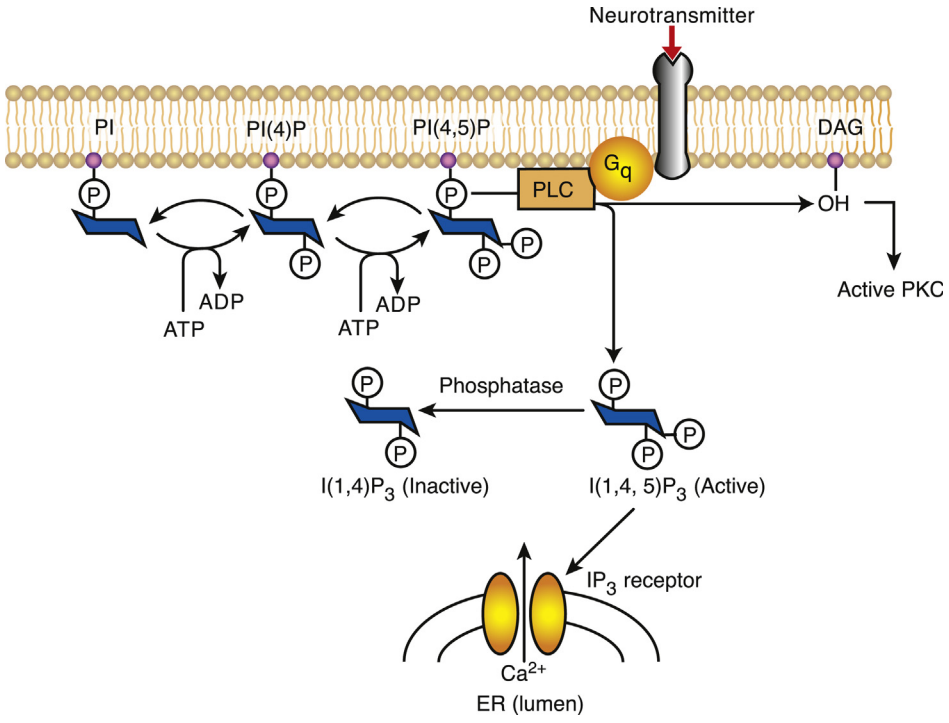

FIGURE 4.7 **Schematic pathway of IP_3 and DAG synthesis and action.** Stimulation of receptors coupled to G_q activates PLC, which leads to release of DAG and IP_3. DAG remains membrane associated and activates PKC, whereas IP_3 diffused into the cell and stimulates the IP_3 receptor in the endoplasmic reticulum (ER), leading to mobilization of intracellular Ca^{2+} stores. IP_3 effects are terminated through dephosphorylation mediated by cellualar phosphatase. *Adapted from Berridge (1993).*

tissues do not contain *de novo* biosynthetic pathways for making inositol. Thus, the phosphatases not only terminate the signal but also serve as a salvage step that may be particularly important when cells are actively undergoing PI turnover. It is intriguing that the simple salt Li^+ selectively inhibits the salvage of inositol by inhibiting the enzyme that dephosphorylates the 1-position and is common to the two pathways. This simple salt is the drug used to treat manic–depressive disorders. At therapeutic doses of Li^+, the reduced salvage of inositol in cells with high phosphoinositide signaling may lead to depletion of PIP_2 and a selective inhibition of this signaling pathway in active cells.

Calcium Ion

Calcium has a dual role as a carrier of electrical current and as a second messenger. Its effects are more diverse than those of other second messengers such as cAMP and DAG because its actions are mediated by a much larger array of proteins, including protein kinases (Carafoli and Klee, 1999). Furthermore, many signaling pathways directly or indirectly increase cytosolic Ca^{2+} concentration from 100 nM to 0.5–1.0 mM. The source of elevated Ca^{2+} can be either the ER or the extracellular space (Fig. 4.8). As indicated earlier, mobilization of ER Ca^{2+} is mediated by IP_3 derived from PLCβ activation through G proteins and from PLCγ activation by receptor tyrosine kinases acting on the IP_3 receptor. In addition, Ca^{2+} can activate its own mobilization through the ryanodine receptor on the ER. Mechanisms for Ca^{2+} influx from outside the cell include several voltage-sensitive Ca^{2+} channels and ligand-gated cation channels that are permeable to

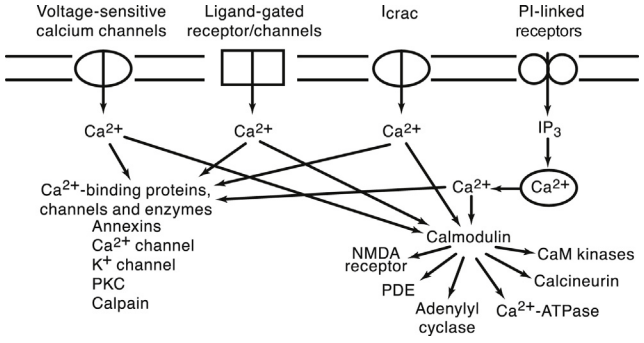

FIGURE 4.8 **Multiple sources of Ca^{2+} converge on calmodulin and other Ca^{2+}-binding proteins.** Cellular levels of Ca^{2+} can rise either by influx (e.g., calcium release-activated calcium current (I_{CRAC}) channels) or by redistribution from intracellular stores triggered by IP_3. Calcium modulates dozens of cellular processes by the action of the Ca^{2+}-calmodulin complex on many enzymes, and calcium has some direct effects on enzymes such as PKC and calpain. CaM kinase, Ca^{2+}-calmodulin-dependent kinase; PDE, phosphodiesterase.

Ca^{2+} [e.g., nicotinic receptor and *N*-methyl-D-aspartate (NMDA) receptor]. In the *Drosophila* visual system and in nonexcitable mammalian cells, depletion of Ca^{2+} from the cytosol and ER initiates an unknown signal that stimulates a low-conductance influx current called I_{CRAC} (Ca^{2+}-release-activated current) that replenishes ER stores.

Dynamics of Ca^{2+} signaling revealed by fluorescent Ca^{2+} indicators. A great deal is known about the spatial and temporal regulation of Ca^{2+} signals because of the development of fluorescent Ca^{2+} indicators. A variety of fluorescent compounds selectively bind Ca^{2+} at physiological concentration ranges and rapidly change their fluorescent properties upon binding Ca^{2+} and report a fairly accurate measurement of free Ca^{2+} (Minta and Tsien, 1989). Digital fluorescence imaging can be used to detect Ca^{2+} in subcellular compartments such as dendrites and spines, the nucleus, and the cytosol and has demonstrated localized changes in free Ca^{2+} (see Chapter 19).

Lack of uniformity in Ca^{2+} levels. The concentration of Ca^{2+} entering the cytosol through voltage-sensitive Ca^{2+} channels in the plasma membrane or through the IP_3 receptor in the ER is extremely high because of the large concentration gradient across these membranes. Relatively low-affinity Ca^{2+}-dependent processes can produce effects of Ca^{2+} near the membrane, such as synaptic release and modulation of Ca^{2+} channels (Chapter 15). However, by the time Ca^{2+} diffuses a few membrane diameters away, it is rapidly buffered by many Ca^{2+}-binding proteins, and its concentration drops from 100 to 1 μM or less (Chapter 6). The diffusion of Ca^{2+} is greatly slowed in biological fluid because of the high concentration of binding proteins (0.2–0.3 mM). Ca^{2+} diffuses a distance of 0.1–0.5 μm, and diffusion lasts approximately 30 ms before Ca^{2+} is bound. Ca^{2+} is therefore a second messenger that acts locally, a feature that makes Ca^{2+} subdomains possible where Ca^{2+} signaling is spatially segregated. In contrast, IP_3 is a global intermediate with an effective range that can span a typical soma before being terminated by dephosphorylation (Allbritton et al., 1992).

Calmodulin-Mediated Effects of Ca^{2+}

Ca^{2+} acts as a second messenger to modulate the activity of many mediators. The predominant mediator of Ca^{2+} action is calmodulin, an abundant and ubiquitous 17-kDa calcium-binding protein. Ca^{2+} binds to calmodulin in the physiological range and converts it into an activator of many cellular targets (Cohen and Klee, 1988). Binding of Ca^{2+} to calmodulin produces a conformational change that greatly increases its affinity for target enzymes. Ca^{2+}-calmodulin binds and activates more than two dozen eukaryotic enzymes, including cyclic nucleotide PDEs, adenylyl cyclase,

nitric oxide synthase, Ca^{2+}-ATPase, calcineurin (a phosphoprotein phosphatase), and several protein kinases (Fig. 4.8). This activation of calmodulin allows neurotransmitters that change the concentration of Ca^{2+} to affect dozens of cellular proteins, presumably in an orchestrated fashion. Ca^{2+} modulates some proteins and enzymes independently of calmodulin (Fig. 4.8).

Calmodulin interacts with its targets in several ways. The "conventional" interaction with enzyme targets requires a stimulated rise in Ca^{2+}. A second mode involves binding at basal Ca^{2+}, to proteins such as GAP-43 (neuromodulin), neurogranin, and unconventional myosins and may serve to localize calmodulin near other targets or to reduce the level of free calmodulin (Persechini and Cronk, 1999). A third group of enzymes, which includes the inducible form of nitric oxide synthase, binds calmodulin in a manner that makes it sensitive to basal Ca^{2+}, and the enzymes are therefore active at basal Ca^{2+}.

Actions of enzymes and proteins modulated by calmodulin. Calmodulin has four Ca^{2+} binding sites, or binding folds, described as EF hands, a recurring Ca^{2+} binding structural motif. Calmodulin is composed of a number of helical segments designated by capital letters and separated by loops. Orientation of the helix−loop−helix EF segment is similar to that of the thumb and index finger, which positions amino acids in the loop for coordination with Ca^{2+}, hence the name EF hand. The ability of Ca^{2+} to be accommodated in an asymmetric coordination shell with multiple and distant amino acids, including uncharged oxygens, enables it to compete with Mg^{2+} and to produce large conformation changes that are the basis for interconversion between inactive and active states of proteins.

Calmodulin recognizes a short segment of the enzymes that it regulates; however, there is no strict consensus sequence for calmodulin binding. X-ray crystallography and NMR show calmodulin to be in an extended structure composed of two globular regions, each containing a set of calmodulin folds separated by a long α-helical tether. Binding of Ca^{2+} allows movement of the globular regions around the calmodulin binding site, "gripping" it as would hands around a rope (Ikura et al., 1992). The two lobes of this compact structure surround the target residues, making dozens of hydrophobic contacts as well as ionic interactions between Arg and Lys typically found in target sequences and Glu residues in calmodulin. This binding likely produces the necessary displacement of the calmodulin binding domain for activation of the target enzymes.

Regulation of guanylyl cyclase by nitric oxide. An important target of Ca^{2+}-calmodulin is the enzyme nitric oxide synthase (NOS). This enzyme synthesizes one of the simplest known messengers, the gas NO (Schmidt and Walter, 1994; Sen and Snyder, 2010). Nitric oxide was first recognized as a signaling molecule that mediates the action of acetylcholine on smooth muscle relaxation (Furchgott and Zawadzki, 1980). In the pathway that led to its discovery, acetylcholine stimulates the PI signaling pathway in the endothelium to increase intracellular Ca^{2+}, which activates NOS so that more NO is made. NO then diffuses radially from the endothelial cells across two cell membranes to the smooth muscle cell, where it activates guanylate cyclase to make cGMP. This in turn activates a cGMP-dependent protein kinase that phosphorylates proteins, leading to a relaxation of muscle. In 1998, Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad received the Nobel Prize for their discoveries concerning nitric oxide as a signaling molecule and therapeutic mediator in the cardiovascular system.

Let us now turn to the details of the NO pathway. We see that other pathways can activate NOS, mediate the actions of NO, stimulate guanylyl cyclases, and mediate the actions of cGMP.

Nitric oxide is derived from L-arginine in a reaction catalyzed by NOS, a complex enzyme that converts L-arginine and O_2 into NO and L-citrulline. NO lasts only a few seconds in biological fluids and thus, no specialized processes are needed to inactivate this particular signaling molecule. As a gas, NO is soluble in both aqueous and lipid media and can diffuse readily from its site of synthesis across the cytosol or cell membrane and affect targets in the same cell or in nearby neurons, glia, and vasculature (Sen and Snyder, 2010). NO produces a variety of effects, including relaxation of smooth muscle of the vasculature, relaxation of smooth muscle of the gut in peristalsis, and killing of foreign cells by macrophages (Schmidt and Walter, 1994). It was first recognized as a neuronal messenger that couples glutamate receptor stimulation to increases in cGMP. Analogs of L-arginine, such as nitroarginine and monomethyl arginine, block NOS unless there is an excess of L-arginine. Such inhibitors of NO synthase have been used to implicate NO in long-term potentiation and long-term depression in the hippocampus and cerebellum, respectively (Schuman and Madison, 1994) (see also Chapter 18).

Three classes of NOS have been characterized. Two of them are constitutively expressed and activated by Ca^{2+}-calmodulin whereas the third is induced by transcription and protein synthesis in response to cell stimulation, e.g., in macrophages stimulated by cytokines. After translation, the inducible form is active at basal Ca^{2+} levels because it has a tightly bound calmodulin in a conformation that enhances its Ca^{2+} sensitivity greatly. The constitutive neuronal isoform is concentrated in cerebellar granule cells and likely provides

the NO that activates guanylyl cyclase in nearby Purkinje cells during the induction of long-term depression in the cerebellum (see Chapter 18).

Activation of guanylyl cyclases. Two types of guanylyl cyclase, a soluble one regulated by NO and a membrane-bound enzyme directly regulated by neuropeptides (Garbers and Lowe, 1994) synthesize cGMP from GTP in a reaction similar to the synthesis of cAMP from ATP. The soluble enzyme is a heterodimer, with catalytic sites resembling those of adenylyl cyclase and a heme group. NO activates the soluble enzyme by binding to the iron atom of the heme moiety. This is the basic mechanism for regulation of soluble guanylyl cyclases. Stimulation of guanylyl cyclase is the major, but not only, effect of NO in the brain and other tissues. A number of therapeutic muscle relaxants, such as nitroglycerin and nitroprusside, are NO donors that produce their effects by stimulating cGMP synthesis.

The membrane-bound guanylyl cyclases are transmembrane proteins with a binding site for neuroendocrine peptides on the extracellular side of the plasma membrane and a catalytic domain on the cytosolic side. Several isoforms of membrane-bound guanylyl cyclase, each with a binding site for a distinct neuropeptide such as atrial natriuretic peptide and brain natriuretic peptide, have been characterized. In the periphery, these peptides regulate sodium excretion and blood pressure; in the brain, their functions are less clear.

Cyclic GMP Phosphodiesterase, an Effector Enzyme in Vertebrate Vision

The versatility of G-protein signaling is illustrated in vertebrate phototransduction, in which a specialized G protein called transducin (G_t) is activated by light rather than by a hormone or neurotransmitter. Without transducin, we would not be able to see. Transducin stimulates cGMP phosphodiesterase, an effector enzyme that hydrolyzes cGMP and ultimately turns off the dark current. Nature has devised an elegant mechanism for using photons of light to modify a hormone-like molecule, retinal, that activates a GPCR called rhodopsin (Baylor, 1996). This receptor has a built-in prehormone that is converted into the active form by light. Light photoisomerizes the inactive 11-*cis*-retinal to the active all-*trans*-retinal, which functions as a transmitter to activate its receptor. Activated rhodopsin triggers the GTP−GDP exchange of transducin, leading to dissociation of its α_t and $\beta\gamma$ subunits. The active species in transducin is the α subunit. It activates a soluble cGMP phosphodiesterase by binding to and displacing an inhibitory subunit of the enzyme. In the dark, retinal rods contain high levels of cGMP, which maintains a cGMP-gated channel permeable to Na^+ and Ca^{2+} in the open state and thus provides a depolarizing dark current. As the levels of cGMP drop, the channel closes to hyperpolarize the cell.

Rods can detect a single photon of light because the signal-to-noise ratio of the system is very low owing to a very low spontaneous conversion of the 11-*cis*-retinal into the all-*trans*-retinal. Furthermore, the amplification factor is quite high; one rhodopsin molecule stimulated by a single photon can activate 500 transducins. Transducin remains in the "on" state long enough to activate 500 PDEs. PDE is designed for speed and can hydrolyze 10^5 cGMP molecules in the second before it is deactivated by GTP hydrolysis and dissociated from transducin (Stryer, 1991). Cyclic GMP in rods regulates a cGMP-gated cation channel, leading to additional amplification of the signal.

Modulation of Ion Channels by G Protein

Each type of neuron has a repertoire of ion channels that give it a distinct response signature, and it is not surprising that several types of mechanisms regulate these channels. Channel modulation occurs via G proteins, second messengers and their cognate protein kinases that phosphorylate ion channels (see also Chapter 16) as well as by direct effects of G proteins.

The first ion channel demonstrated to undergo regulation by G proteins was the cardiac K^+ channel that mediates slowing of the heart by acetylcholine released from the vagus nerve. When this I_{KACh} channel is examined in a membrane patch delimited by the seal of a cell-attached electrode, addition of acetylcholine within the electrode increases the frequency of channel opening, whereas addition of acetylcholine to the cell surface outside the seal does not. Although acetylcholine stimulates muscarinic M2 receptors when added either inside or outside the seal, the receptors outside do not have access to the channels being recorded in the sealed patch because this signaling pathway does not include diffusible second messengers that can affect distant channels. The process is therefore described as membrane delimited, which is explained most simply by a direct interaction between the G protein and the channel. Subsequent studies have shown that the pathway is pertussis toxin sensitive and that purified G_i activated by GTPγS added to the underside of the patch will activate the channel.

The channel is activated by G_i but whether the active component of G_i is the α or the $\beta\gamma$ subunit was controversial. Dogmas do not die easily and, for many years, $\beta\gamma$ was not considered a direct activator or inhibitor of effector enzymes and channels. It turns out that the I_{KACh} channel can be activated either by α_i or by $\beta\gamma$ (Huang et al., 1995; Wickman et al., 1994).

Ca^{2+} channels are a common target of regulation by neurotransmitters and some of this occurs via G

proteins. In the heart, where L-type Ca^{2+} channels are critical for regulation of contractile strength, the Ca^{2+} current is enhanced by α_s formed by β-adrenergic stimulation of G_s. In contrast, N-type Ca^{2+} channels, which modulate synaptic release in nerve terminals, are often inhibited by muscarinic and α-adrenergic agents and by opiates acting at receptors coupled to G_i and G_o. In sympathetic ganglia, norepinephrine reduces synaptic release by inhibiting Ca^{2+} influx through the N channel by favoring the time spent in a low-open probability mode (Delcour and Tsien, 1993).

G-Protein Signaling Gives Special Advantages in Neural Transmission

The G protein-based signaling system provides several advantages over fast transmission (Hille, 1992; Birnbaumer, 2007). These advantages include amplification of the signal, modulation of cell function over a broad temporal range, diffusion of the signal to a large cellular volume, cross-talk, and coordination of diverse cell functions.

Amplification

Several thousandfold amplification can be initiated by a single neurotransmitter−receptor complex that activates numerous G proteins, each of which activates many effector enzymes and channels. Each enzyme can generate many second−messenger molecules, and each channel allows the flux of many ions. As we see in the next section, second messengers often activate protein kinases that phosphorylate many substrates before deactivation.

Temporal Range

The sacrifice in speed relative to signaling by ligand-gated ion channels is compensated by a broad range of signaling that facilitates integration of signals by the G-protein system. Transmission through membrane-delimited coupling of ion channels to G proteins is relatively fast, with only some sacrifice in speed. Signaling that includes second messengers is much slower. It can be as fast as 100−300 ms, as in olfactory signaling in which cAMP and IP_3 take part, or it can take from seconds to minutes.

Spatial Range

A slower time frame means that cellular processes that are quite distant from the receptor can be modulated. Diffusion of second messengers such as IP_3, Ca^{2+}, and DAG can extend neurotransmission through the cell body and to the nucleus to alter gene expression.

Cross-Talk

Both the signal transduction machinery and the ultimate mediators of their responses, such as the protein kinases, are capable of cross-talk. This is seen in coincident detection of signals from two receptors converging on AC1 and AC2 adenylyl cyclase.

Coordinated Modulation

Neurotransmitters acting through G proteins can elicit a coordinated response of the cell that can modulate synaptic release, resynthesis of neurotransmitter, membrane excitability, the cytoskeleton, metabolism, and gene expression.

Summary

A major class of signaling using G protein-linked signals affords the nervous system a rich diversity of modulation, amplification, and plasticity. Signals are mediated through second messengers activating proteins that modify cellular processes and gene transcription. A key feature is the ability of G proteins to detect the presence of activated receptors and to amplify the signal through effector enzymes and channels. Phosphorylation of key intracellular proteins, ion channels, and enzymes activates diverse, highly regulated cellular processes. Specificity of response is ensured through receptors reacting only with a limited number of G proteins. The response of the system is determined by the speed of activation of GTPase. In addition to speed of response, the spatial compartmentalization of the system enables specificity and localized control of signaling. Phospholipids and phosphoinositols are also substrates for second messenger signaling for G proteins. Stimulation of release of intracellular calcium is often the mediator of the signal. Calcium itself has a dual role as a carrier of electrical current and as a second messenger. Calmodulin is a key regulator that provides complexity and enhances specificity of the signaling system. Sensitivity of the system is imparted by an extremely robust amplification system, as seen in the visual system, which can detect single photons of light.

MODULATION OF NEURONAL FUNCTION BY PROTEIN KINASES AND PHOSPHATASES

Protein phosphorylation and dephosphorylation are key processes that regulate cellular function. They play a fundamental role in mediating signal transduction initiated by neurotransmitters, neuropeptides, growth factors, hormones, and other signaling molecules. The primary determinants of morphology and function of a cell are the protein constituents expressed in that cell. However, the functional state of many of

these proteins is modified by phosphorylation–dephosphorylation, the most ubiquitous posttranslational modification in eukaryotes. More than a fifth of all proteins serve as targets for kinases and phosphatases. Phosphorylation or dephosphorylation can rapidly modify the function of enzymes, structural and regulatory proteins, receptors, and ion channels taking part in diverse processes without a need to change the level of their expression. As is described in greater detail in the following chapter, phosphorylation and dephosphorylation can also produce long-term alterations in cellular properties by modulating transcription and translation and changing the complement of proteins expressed by cells.

Protein kinases catalyze the transfer of the terminal, or γ, phosphate of ATP to the hydroxyl moieties of Ser, Thr, or Tyr residues at specific sites on target proteins. Most protein kinases are either Ser/Thr kinases or Tyr kinases, with only a few designed to phosphorylate both categories of acceptor amino acids. Protein phosphatases catalyze the hydrolysis of the phosphoryl groups from phosphoserine–phosphothreonine, phosphotyrosine, or both types of phosphorylated amino acids on phosphoproteins.

Regulation of the phosphorylation state of proteins is bidirectional, involving both kinases and phosphatases (Fig. 4.9). The phosphorylation state can be dynamically altered either upward or downward from the steady state, depending on the cell's inputs and its complement of kinases and phosphatases. The phosphorylation state of proteins *in vivo* ranges widely, from minimal to almost fully phosphorylated, even in the absence of cell stimulation (Rosenmund et al., 1994; Greengard et al., 1998).

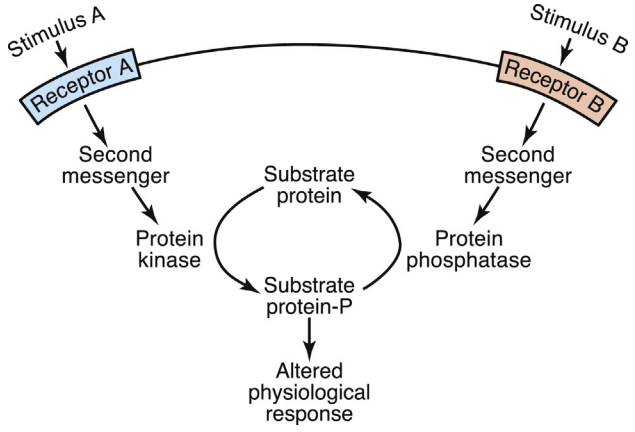

FIGURE 4.9 **Regulation by protein kinases and protein phosphatases.** Enzymes and other proteins serve as substrates for protein kinases and phosphoprotein phosphatases, which modify their activity and control them in a dynamic fashion. Multiple signals can be integrated at this level of protein modification. *Adapted from Greengard et al. (1998).*

The activity of protein kinases and protein phosphatases is typically regulated either by a second messenger (e.g., cAMP or Ca^{2+}) or by an extracellular ligand (e.g., nerve growth factor). In general, the second messenger-regulated kinases modify Ser and Thr, whereas the receptor-linked kinases modify Tyr. Among the hundreds of protein kinases and protein phosphatases in neurons, a relatively small number serve as master regulators to orchestrate neuronal function.

The cAMP-dependent protein kinase (PKA) is a prototype for the known regulated Ser/Thr kinases; they are similar in overall structure and regulatory design. PKA is exemplified here because the experimental strategies currently being used in the study of kinases have come from the investigation of PKA-mediated processes. As its name implies, PKA carries out the posttranslational modification of numerous protein targets in response to signal transduction processes that act through G proteins and alter the level of cAMP in cells. PKA is the predominant target or mediator for signaling through cAMP, the only others being a cAMP-liganded ion channel in olfaction and the "exchange protein directly activated by cAMP" termed Epac, which modulates GDP-GTP exchange for the small GTPases Rap1 and Rap2. Epacs are activated by cAMP indpendent of PKA action and regulate cell adhesion, cell-junction formation, and exocytosis (Bos, 2006).

In a similar fashion, the related cGMP-dependent protein kinase (PKG) mediates most of the actions of cGMP. Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) and several other kinases mediate many of the actions of stimuli that elevate intracellular Ca^{2+}. Finally, the PI signaling system increases both DAG and Ca^{2+}, which activate any of a family of protein kinases collectively called protein kinase C (PKC). Each of these kinases has a broad substrate specificity and is therefore able to phosphorylate diverse substrates throughout the cell.

Phosphorylation and dephosphorylation are reversible processes, and the net activity of the two processes determines the phosphorylation state of each substrate. Among the many phosphoprotein phosphatases, a relatively small number exemplified by protein phosphatase 1 (PP-1), protein phosphatase 2A (PP-2A), and protein phosphatase 2B (PP-2B, or calcineurin) are responsible for most of the dephosphorylation at Ser and Thr residues on phosphoproteins that are under the regulation of the afore- mentioned kinases. The Nobel Prize for Physiology or Medicine was awarded to Edwin Krebs and Edmond Fischer in 1992 for their pioneering work on regulation of cell function by protein kinases and phosphatases.

Certain Principles are Common in Protein Phosphorylation and Dephosphorylation

Protein kinases and protein phosphatases are described either as multifunctional, if they have a broad specificity and therefore modify many protein targets, or as dedicated, if they have a very narrow substrate specificity and may modify only a single protein target. The Ser/Thr kinases and phosphatases described here are multifunctional, but how is response specificity achieved with kinases and phosphatases that are designed to recognize many substrates? These enzymes are by no means promiscuous; their substrates conform either to a consensus sequence along the primary protein sequence (for the kinases) or to general features of the three-dimensional structure of the phosphoprotein (for the phosphatases). Spatial positioning of kinases and their substrates in the cell further determines the likelihood of phosphorylation–dephosphorylation of a given substrate.

The amplification of signal transduction described earlier is continued during the transmission of the signal by protein kinases and protein phosphatases. In some cases, the kinases are themselves subject to activation by phosphorylation in a cascade in which one activated kinase phosphorylates and activates a second, and so on, to provide amplification and a switchlike response termed *ultrasensitivity* (Ferrell and Machleder, 1998) (see Chapter 6 for additional discussion of ultrasensitivity).

Kinases and phosphatases integrate cellular stimuli and encode the stimuli as the steady-state level of phosphorylation of a large complement of proteins in the cell (Hunter, 1995). The phosphorylation state depends on the degree of activation or inactivation of the protein kinase or protein phosphatase, the affinity of the protein target for these enzymes, and the concentration and access of the kinase, phosphatase, and target protein. Some proteins are phosphorylated largely in the basal state and are subject primarily to regulation of phosphatases. Distinct signal transduction pathways can converge on the same or different target substrates. In some cases, these substrates can be phosphorylated by several kinases at distinct or overlapping sites.

Phosphorylation produces specific changes in the function of a target protein, such as increasing or decreasing the catalytic activity of an enzyme, the affinity of a protein with DNA, phospholipids, or other cellular constituents, and desensitization or localization of receptors. Any of several characteristics of ion channels can be altered by phosphorylation, including voltage dependence, probability of being opened, open and close time kinetics, and conductance. The number of possible effects is almost limitless and enables the fine-tuning of numerous cellular processes over broad time scales, from milliseconds to hours. Kinases and phosphatases do this fine-tuning by regulating the presence of a highly charged and bulky phosphoryl moiety on Ser, Thr, or Tyr at a precise location on the substrate protein. The phosphate may introduce a steric constraint at the surface of the protein in interactions with other cellular constituents, or the negative charge of the phosphoryl moiety may elicit a conformational change because of attractive or repulsive ionic interactions between the phosphorylated segment and other charged amino acids on the protein.

Finally, each of the three kinases exemplified here is capable of functioning as a cognitive kinase, that is, a kinase capable of a molecular memory. Although the activity of each kinase requires a second messenger for initial activation, each can become persistently active and independent of its second messenger and initiating stimulus. As is described in greater detail in Chapter 18, this molecular memory potentiates the activity of these kinases and may enable them to participate in aspects of neuronal plasticity.

cAMP-Dependent Protein Kinase was the First Well-Characterized Kinase

Neurotransmitters that stimulate the synthesis of cAMP exert their intracellular effects primarily by activating PKA (Nairn et al., 1985). The functions (and substrates) regulated by PKA include gene expression (cAMP response element-binding protein, or CREB), catecholamine synthesis (tyrosine hydroxylase), carbohydrate metabolism (phosphorylase kinase), cell morphology (microtubule-associated protein 2, or MAP-2), postsynaptic sensitivity (AMPA receptor), and membrane conductance (K^+ channel). Paul Greengard and Eric Kandel received the Nobel Prize for Physiology or Medicine in 2000 (along with Arvid Carlsson) for their discoveries on neuromodulation via PKA and phosphoprotein phosphatases.

PKA is a tetrameric protein composed of two types of subunits: (1) a dimer of regulatory (R) subunits (either two RI subunits for Type I PKA or two RII subunits for Type II PKA) and (2) two catalytic subunits (C subunit) (Scott, 1991). Two or more isoforms of the RI, RII, and C subunits have distinct tissue and developmental patterns of expression but appear to function similarly. The C subunits are 40-kDa proteins that contain the binding sites for protein substrates and ATP. The R subunits are 49- to 51-kDa proteins that contain two cAMP binding sites. In addition, the R subunit dimer contains a region that interacts with cellular anchoring proteins that serve to localize PKA appropriately within the cell.

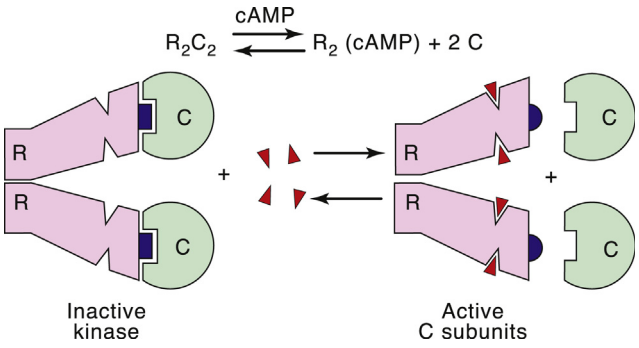

FIGURE 4.10 **Activation of PKA by cAMP.** An autoinhibitory segment (blue) of the regulatory subunit (R) dimer (pink) interacts with the substrate binding domain of the catalytic (C) subunits of PKA (green), blocking access of substrates to their binding site. Binding of four molecules of cAMP (small red triangles) reduces the affinity of R for C, resulting in dissociation of constitutively active C subunits.

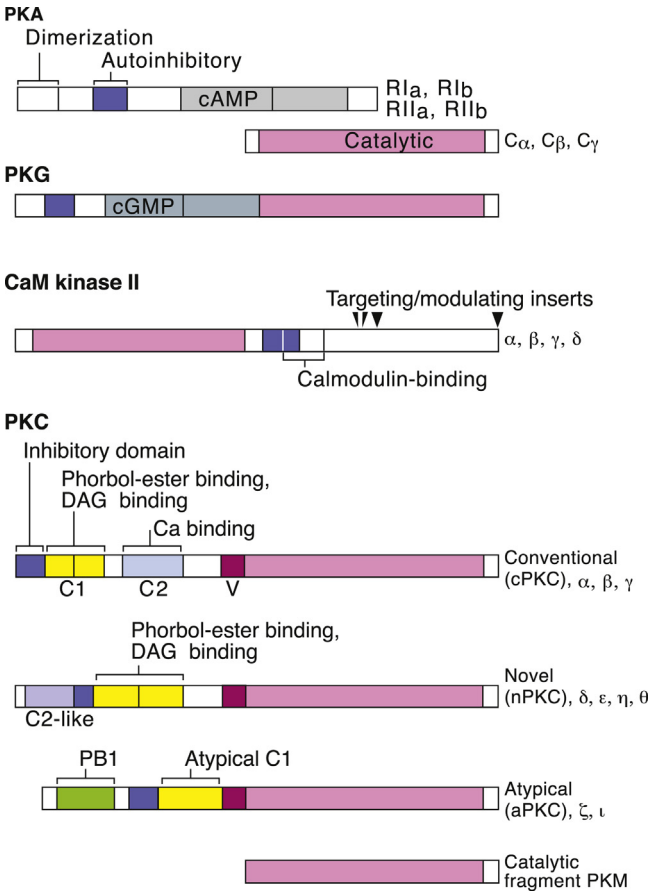

FIGURE 4.11 **Domain structure of protein kinases.** Protein kinases are encoded by proteins with recognizable structural sequences that encode specialized functional domains. Each of the kinases [PKA, PKG, CaM (Ca^{2+}-calmodulin-dependent) kinase II, and PKC] have homologous catalytic domains (pink) that are kept inactive by the presence of an autoinhibitory segment (blue segments). The regulatory domains contain sites for binding second messengers such as cAMP, cGMP, Ca^{2+}-calmodulin, DAG, and Ca^{2+}-phosphatidylserine. Alternative splicing creates additional diversity.

The binding of second messengers by PKA and the other second messenger-regulated kinases relieves an inhibitory constraint and thus activates the enzymes (Fig. 4.10). The C subunit has intrinsic protein kinase activity that remains inhibited as long as the C subunit is complexed with the R subunits in the tetrameric holoenzyme. Cyclic AMP activates the C subunit by facilitating dissociation of its inhibitory R subunits. The steady-state level of cAMP determines the fraction of PKA that is in the dissociated or active form. In this way PKA decodes cAMP signals into the phosphorylation of proteins and the resultant change in various cellular processes.

PKA is a member of a large family of protein kinases that have in common a significant degree of homology in their catalytic domains and are likely derived from an ancestral gene (Hanks and Hunter, 1995) (Fig. 4.11). This homology extends to the three-dimensional crystal structure, based on X-ray crystallography of PKA and a few other kinases. The catalytic domain may be in a subunit distinct from the regulatory domain, as in PKA, or in the same subunit, as in PKC and Ca^{2+}-calmodulin-dependent (CaM) kinases. The crystal structure of the C subunit complexed to a segment of protein kinase inhibitor (PKI), a selective high-affinity inhibitor of PKA, reveals that the C subunit is composed of two lobes (Knighton et al., 1991). A small N-terminal lobe contains a highly conserved region that binds Mg^{2+}-ATP in a cleft between the two lobes. A larger C-terminal lobe contains the protein–substrate recognition sites and the appropriate amino acids for catalyzing the transfer of the γ-phosphoryl moiety from ATP to the polypeptide chain of the substrate. Inhibition by PKI is diagnostic of PKA involvement; PKI contains an autoinhibitory sequence resembling PKA substrates and is positioned in the catalytic site like a substrate, thus blocking access for substrates.

How can protein kinases utilize a homologous structure yet exhibit phosphorylation target specificity? Although the C-terminal lobes of all kinases may use a similar scaffold for their peptide binding and catalytic sites, distinct amino acids are positioned on this scaffold to produce specificity in peptide binding. Some substrates also have a distinct docking site for the kinase that localizes the kinase and serves to further increase the selectivity of phosphorylation (Smith et al., 2000).

PKA phosphorylates Ser or Thr at specific sites in dozens of proteins. The sequences of amino acids at the phosphorylation sites are not identical, but a consensus sequence can be deduced from a comparison of these sequences (Kennelly and Krebs, 1991). PKA

phosphorylates at sites with the consensus sequence Arg−Arg−X−Ser/Thr−Y, in which X can be one of many different amino acids and Y is a hydrophobic amino acid. Each kinase has a characteristic consensus sequence that forms the basis for distinct substrate specificities although many substrates are phosphorylated at "anomalous" sites (Walsh and Patten, 1994). The consensus sites of PKA, CaMKII, and PKC all include a basic residue on the substrate, and these kinases do share some target substrates.

A regulatory theme common to PKA, CaMKII, and PKC is that their second messengers activate them by displacing their respective inhibitory domains from the active site; that is, they activate by relieving an inhibitory constraint (Kemp et al., 1994). In PKA, binding of the R subunit distorts the active site of the C subunit and blocks access of substrates by positioning an inhibitory sequence in the catalytic site. This segment of R binds to C as would a substrate or PKI (protein kinase inhibitor). Binding of cAMP to the R subunit produces a large conformation change in R that disrupts its binding to the C subunit, thus leading to dissociation of an active C subunit (Su et al., 1995). CaMKII and PKC have the catalytic domain and inhibitory sequence in the same polypeptide with binding of their respective second-messengers similarly de-inhibiting the kinase by displacement of the autoinhibitory domain (Kemp et al., 1994) (see Fig. 4.11).

Functional differences between Type I and Type II PKA (which have C subunits in common but have different R subunits) may arise from differential targeting in cells and from differences in regulation by autophosphorylation. RII, but not RI, is autophosphorylated by its C subunit when it is in the holoenzyme form. This potentiates cAMP action by reducing the rate of reassociation of RII and C after a stimulus. Only anchoring proteins for RII have been characterized thus far (Beene and Scott, 2007).

Multifunctional CaMKII Decodes Diverse Signals that Elevate Intracellular Ca^{2+}

Most of the effects of Ca^{2+} in neurons and other cell types are mediated by calmodulin, and many of the effects of Ca^{2+}-calmodulin are mediated by protein phosphorylation−dephosphorylation. In contrast with the cAMP system, both dedicated and multifunctional kinases are found in the Ca^{2+} signaling system (Wayman et al., 2011). Two kinases, MLCK and phosphorylase kinase, are each dedicated to the phosphorylation of a single substrate—myosin light chains and phosphorylase, respectively. The Ca^{2+} signaling system also contains a family of Ca^{2+}-calmodulin-dependent protein kinases with broad substrate specificity, including CaMKI, CaMKII, and CaMKIV. CaMKII phosphorylates tyrosine hydroxylase, MAP-2, synapsin I, calcium channels, Ca^{2+}-ATPase, transcription factors, and glutamate receptors and thereby regulates synthesis of catecholamines, cytoskeletal function, synaptic release in response to high-frequency stimuli, calcium currents, calcium homeostasis, gene expression, and synaptic plasticity, respectively. The enzyme is activated by Ca^{2+} influx or release from intracellular stores. It is found in every tissue but is particularly enriched in neurons, where it may account for as much as 2% of all hippocampal protein. It is found in the cytosol, in the nucleus, in association with cytoskeletal elements, and in postsynaptic thickening, termed the postsynaptic density, which is found in asymmetric synapses. CaMKII is a large multimeric enzyme, consisting of 12 subunits derived from four homologous genes (α, β, γ, and δ) that encode different isoforms of the kinase that range from 54 to 72 kDa per subunit. Multimers and heteromultimers of α- and β-CaMKII isoforms are found predominantly in brain, whereas the γ- and δ-CaMKII are found throughout the body, including the brain.

The catalytic, regulatory and targeting/association domains of CaMKII are all contained within a single polypeptide (Fig. 4.11). The N-terminal half of each isoform contains the catalytic domain, which is highly homologous to the catalytic subunit of PKA and other Ser/Thr kinases. The middle region constitutes the regulatory domain, which contains an autoinhibitory domain with an overlapping calmodulin-binding sequence. The C-terminal end contains an association domain that allows 12 subunits (two rings of six subunits) to assemble into a multimer (Chao et al., 2011), as well as target sequences that direct the kinase to distinct intracellular sites.

Regulation of the kinase by autophosphorylation is a critical feature of CaMKII. The kinase is inactive in the basal state because an autoinhibitory segment distorts the active site and sterically blocks access to its substrates (Rellos et al., 2010). Binding of Ca^{2+}-calmodulin to the calmodulin-binding domain displaces the autoinhibitory domain from the catalytic site thus activating the kinase by enabling a more active conformation and binding of protein substrates and ATP. The activated kinase autophosphorylates Thr-286 (in α-CaMKII and comparable Thr on other isoforms). Phosphorylation of this site disables the autoinhibitory segment by preventing it from reblocking the active site after calmodulin dissociates and thereby locks the kinase in a partially active state that is independent, or autonomous, of Ca^{2+}-calmodulin (Saitoh and Schwartz, 1985; Miller and Kennedy, 1986; Hanson et al., 1989) and can anchor to additional targets. Displacement of this domain also exposes a binding

site for anchoring proteins that the activated kinase can bind (Bayer et al., 2001). Interestingly, such binding to both the NMDA receptor and eag K^+ channel leads to a persistently active kinase without the need for autophosphorylation and may do so by similarly keeping the autoinhibitory domain from reblocking the active site (Bayer et al., 2001; Sun et al., 2004).

An additional dramatic effect of autophosphorylation is that it enhances the affinity of the bound calmodulin by 400-fold, which it achieves by reducing the rate of dissociation of calmodulin from the kinase after Ca^{2+} levels are reduced below threshold. In essence, autophosphorylation traps bound calmodulin for several seconds and keeps the kinase active for a while after Ca^{2+} levels decline to baseline. The consequence of calmodulin trapping and disruption of the autoinhibitory domain is to prolong the active state of the kinase, a potentiation that led to its description as a cognitive kinase (Schulman and Braun, 1999; Lisman, 1994). Calmodulin trapping also prolongs the residence time of activated kinase that translocates to synaptic sites.

CaMKII is targeted to distinct cellular compartments. Differences between the four genes encoding CaMKII and between the two or more isoforms that are encoded by each gene by apparent alternative splicing reside primarily in a variable region at the start of the association domain (see Fig. 4.11). In some isoforms, this region contains additional amino acids that target those isoforms to the nucleus. The major neuronal isoform, α-CaMKII, is largely cytosolic but is also found attached to postsynaptic densities and to synaptic vesicles and may therefore have several targeting sequences. Targeting to the NMDA type glutamate receptor and to the eag K^+ channel occurs only after calmodulin activates the kinase and exposes a binding site (Bayer et al., 2001; Sun et al., 2004). Calmodulin trapping prolongs the residence time of kinase translocated to synaptic sites. The kinase also serves in a noncatalytic capacity as a scaffold for binding of other proteins, including the recruitment of the ubiquitin proteasome and the immediate early gene Arc/Arg3.1 to dendritic spines.

Protein Kinase C is the Principal Target of the PI Signaling System

PKC is the name for members of a relatively diverse family of protein kinases most closely associated with the PI signaling system. PKC is a multifunctional Ser/Thr kinase capable of modulating many cellular processes, including exocytosis and endocytosis of neurotransmitter vesicles, neuronal plasticity, gene expression, regulation of cell growth and cell cycle, ion channels, and receptors. The role of DAG generated during PI signaling was unclear until its link to PKC was established (Takai et al., 1979; Tanaka and Nishizuka, 1994). Many PKC isoforms also require an acidic phospholipid such as phosphatidylserine for appropriate activation. The kinase is also of interest because it is the target of a class of tumor promoters called phorbol esters. They activate PKC by simulating the action of DAG, bypassing the normal receptor-based pathway and inappropriately stimulating cell growth.

The PKC family of kinases is diverse in structure and regulatory properties (Newton, 2010; Rosse et al., 2010). PKC is a monomeric enzyme (78−90 kDa) with catalytic, regulatory, and targeting domains all on one polypeptide. Each isoform has a regulatory domain, with several subdomains, in its N-terminal half and a catalytic domain at the C terminal (see Fig. 4.11). Only the first PKC isoforms to be characterized, now termed the conventional isoforms (or cPKC), have all of the following domains: (1) an autoinhibitory or pseudosubstrate sequence; (2) C1, a cysteine rich domain that binds DAG and phorbol esters; (3) C2, a region necessary for Ca^{2+} sensitivity and for binding to phosphatidylserine and to anchoring proteins; (4) V, a variable protease-sensitive hinge; and (5) the catalytic domain.

Another class of isoforms, termed novel PKCs (nPKC), lacks a true C2 domain and is therefore not Ca^{2+} sensitive. Another class is considered atypical (aPKC) because it lacks C2 and the first of two cysteine-rich domains that are necessary for DAG (or phorbol ester) sensitivity. This class is neither Ca^{2+} nor DAG sensitive but has a PB1 domain involved in protein interaction. Not included is a DAG-interacting kinase originally designated as PKCμ and now termed PKD because its catalytic domain is different from the other PKC isoforms.

Activation of PKC is best understood for the conventional isoforms. Generation of DAG (specifically its *sn*-1,2-diacylglycerol isomer) resulting from stimulation of the PI signaling pathway increases the affinity of cPKC isoforms for Ca^{2+} and phosphatidylserine. Cell stimulation results in the translocation of cPKC from a variety of sites to the membrane or cytoskeletal elements where it interacts with PS-Ca^{2+}-DAG at the membrane (Newton, 2010; Kraft and Anderson, 1983; Zhang et al., 1995; Ron et al., 1994). Binding of the second messengers to the regulatory domain disrupts the nearby autoinhibitory domain, leading to a reversible activation of PKC by deinhibition (Muramatsu et al., 1989).

Translocation is not restricted to the plasma membrane. Upon activation some PKC isoforms translocate reversibly to intracellular sites enriched with certain anchoring proteins for the activated form of PKC,

termed RACK (receptors for activated C kinase) (Mochly-Rosen, 1995; Ron et al., 1994). Activation may consist of both displacement of the autoinhibitory segment to unblock the catalytic site and displacement of an "auto-anchor" site to unblock the RACK binding site.

Prolonged activation of PKC can be produced by the addition of phorbol esters, which simulate activation by DAG but remain in the cell until they are washed out. In a matter of hours to days, such persistent activation by phorbol esters leads to a degradation of PKC. This phenomenon is sometimes used experimentally to produce a PKC-depleted cell (at least for phorbol ester binding isoforms) and thereafter to test for a loss of putative PKC functions.

Spatial Localization Regulates Protein Kinases and Phosphatases

Protein kinases and protein phosphatases are often localized near their substrates or they translocate to their substrates upon activation to improve speed and specificity in response to neurotransmitter stimulation. PKA is targeted to intracellular sites on the cytoskeleton, membrane, and Golgi through interactions between the RII subunit and specific anchoring proteins (Logue and Scott, 2010). One of its anchoring proteins, A Kinase Anchoring Protein 79 (AKAP79) is an anchor for PKA, for PKC, and for calcineurin, the Ca^{2+}-calmodulin-dependent phosphatase. Better coordination of the phosphorylation−dephosphorylation of the same or different substrates may be achieved by placing calcineurin, PKC, and PKA in the same compartment through AKAPs. Another example of a signaling complex is the protein termed *yotiao*, which binds to the NMDA-type glutamate receptor and serves as an anchor for both PKA and a phosphatase (PP-1) (Westphal et al., 1999).

The use of anchoring proteins has several consequences. First, rate of phosphorylation and specificity are enhanced when kinases or phosphatases are concentrated near intended substrates. Second, it increases the signal-to-noise ratio for substrates that are not near anchoring proteins by reducing basal state phosphorylation. For example, PKA is anchored on the Golgi away from the nucleus so that phosphorylation in the basal state or even after a brief stimulus produces little phosphorylation of nuclear proteins. Prolonged stimuli, however, enable some C subunits to passively diffuse through nuclear pores and into nuclei, where they can participate in regulation of gene expression (Bacskai et al., 1993). Termination of the nuclear action of C subunits is aided by PKI, which inhibit the enzyme and export it back out of the nucleus (Wen et al., 1995). Third, concentration of kinases via anchoring enables significant phosphorylation of nearby substrates even at low level of kinase activation, such as in the basal state. In such a situation the regulation of the substrate would occur by pathways that modulate its phosphatase or that modify the exposure of the phosphorylation site on the substrate to the nearby kinase.

PKA, CaMKII, and PKC are Cognitive Kinases

The ability of three major Ser/Thr kinases (PKA, CaMKII, and PKC) in brain to initiate or maintain synaptic changes that underlie learning and memory (Chapters 18 and 20) may require that they themselves undergo some form of persistent change in activity. As mentioned earlier, they have been described as cognitive kinases because they are capable of sustaining their activated states after their second messengers return to basal level and because their target substrates modulate synaptic plasticity (Schwartz, 1993).

cAMP-Dependent Protein Kinase

As is discussed in greater detail in Chapter 18, a role for PKA as a cognitive kinase can be seen in long-term facilitation of the withdrawal reflexes in *Aplysia* and in long-term potentiation in the vertebrate hippocampus. In *Aplysia*, stimulation of the body with a strong stimulus such as an electric shock facilitates the withdrawal response to a light touch delivered to another part of the animal. This is an example of a simple form of learning called sensitization. A single shock produces a short-lasting memory, but repeated shocks (training) produce a memory that can last several days. The shock stimulates the release of serotonin, which increases cAMP and PKA activity in the sensory neurons. Features of the behavioral training can be simulated in a system in which a single sensory neuron is co-cultured with a single motor neuron and serotonin is applied to the bath to mimic the effects of sensitizing stimuli. A single exposure to serotonin (or cAMP) produces short-term facilitation and a short-term increase in the phosphorylation of more than a dozen PKA substrates in these cells. However, repeated or prolonged exposure to these agents leads to long-term facilitation and an enhanced state of phosphorylation of the same set of proteins. This phenomenon is due to a PKA that is persistently active despite the fact that cAMP is no longer elevated (Chain et al., 1999). A possible scheme for this phenomenon is shown in Fig. 4.12 (see also Fig. 19.24). Phospho-RII and C subunits dissociate on elevation of cAMP and the rate of reassociation following the decrease in cAMP levels is greatly reduced by the

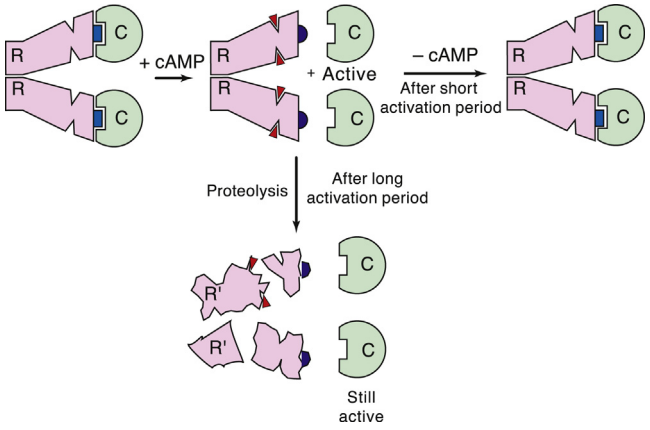

FIGURE 4.12 **Long-term stimulation can convert PKA into a constitutively active enzyme.** Dissociation of PKA R (pink) and C (green) subunits is reversible with short-term elevation of cAMP (small red triangles). More prolonged activation results in loss of R subunits to proteolysis, resulting in an insufficient amount of R subunits to associate with and inhibit all C subunits after the cAMP stimulus terminates.

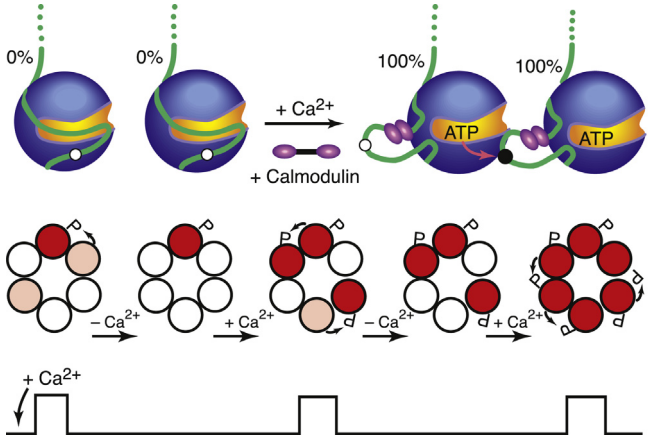

FIGURE 4.13 **Frequency-dependent activation of CaMKII.** Autophosphorylation occurs when both of two neighboring subunits in a holoenzyme are bound to calmodulin. At high frequency of stimulation (rapid Ca^{2+} spikes), the interspike interval is too short to allow significant dephosphorylation or dissociation of calmodulin, thereby increasing the probability of autophosphorylation with each successive spike. In a simplified CaMKII with only six subunits, calmodulin-bound subunits are shown in pink, and autophosphorylated subunits with trapped calmodulin are shown in red. *Adapted from Hanson and Schulman (1992).*

presence of phosphate on the R subunit, thus prolonging phosphorylation of various target proteins by the C subunit. The R subunits are more susceptible than the C subunits to proteolytic degradation in their dissociated state and thus prolonged or repetitive stimulation leads to a preferential decrease in the inhibitory R subunits. At the end of the stimulation a slight excess of C subunits remain persistently active because of insufficient R subunits. The various targets of PKA can then be phosphorylated by this active C subunit long after cAMP levels return to basal or prestimulus levels. Prolonged activation of PKA enables the C subunit to enter the nucleus and induce gene expression, and one of these genes facilitates further proteolysis of R. Although short-term facilitation does not require transcription of new genes or protein synthesis, the long-term effects on phosphorylation and on facilitation do require transcription of new genes and protein synthesis (Kaang et al., 1993). In this interesting process, a molecular memory of appropriate stimulation by serotonin is encoded by a persistence of PKA activity that is regenerative.

Ca^{2+}-Calmodulin-Dependent Protein Kinase

CaMKII has features of a cognitive kinase because it has a molecular memory of its activation that is based on autophosphorylation and it phosphorylates proteins that modulate synaptic plasticity (Coultrap and Bayer, 2012; Lisman, et al., 2012). The biochemical properties of CaMKII suggest mechanisms by which appropriate stimulus frequencies can generate an autonomous enzyme (Fig. 4.13; see also Fig. 6.3 in Chapter 6). Autophosphorylation takes place within each holoenzyme but requires the phosphorylation of one subunit by a proximate neighbor. Individual stimuli may be too brief and available calmodulin may be limited so a single stimulus would active only a few subunits per holoenzyme. At low stimulus frequency, the time between stimuli is sufficient for calmodulin to dissociate and the kinase to be dephosphorylated, and the same submaximal activation will occur with each stimulus. Brief stimuli at low frequency produce minimal autophosphorylation because autophosphorylation only occurs when two proximate neighboring subunits are simultaneously activated apparently because Ca^{2+}/calmodulin binding exposes the phosphorylation site on the "substrate" subunit (Hanson et al, 1994). However, at higher frequencies, some subunits will remain autophosphorylated and bound to calmodulin so successive stimuli will result in more calmodulin bound per holoenzyme, which will make autophosphorylation more probable. The enzyme is therefore able to decode the frequency of cellular stimulation and translate this into a prolonged activated state. Low-frequency stimulation leads to submaximal activation of the kinase at each stimulus, whereas higher frequencies exceed a threshold beyond which stimulation leads to recruitment of additional calmodulin and a higher level of activation and autonomy with each spike (De Koninck and Schulman, 1998). See Chapter 6 for additional discussion of the dynamics and effects of CaMKII autophosphorylation.

CaMKII phosphorylates a number of substrates that affect synaptic strength and its autophosphorylation is critical for memory. An α-CaMKII mouse knock-in in which the critical Thr was replaced by Ala blocks autonomy and the induction of long-term potentiation (Giese et al., 1998). These mice are deficient in learning spatial navigational cues, one of the functions of the rodent hippocampus. The basis for its role in synaptic strength and memory involves a net shift of AMPA receptors to the synapse that may be mediated by phosphorylation of both GluA1 receptors and their accessory protein(s), leading to a greater postsynaptic response (Lisman et al., 2012; Lu et al., 2010; Opazo et al., 2010). The enzyme can therefore be appropriately described as a cognitive kinase with respect to its own molecular memory as well as its functional role in mediating aspects of synaptic plasticity.

Protein Kinase C

PKC also can be converted into a form that is independent, or autonomous, of its second messenger and can be described as a cognitive kinase. Before Ca^{2+} and DAG were known to have roles in the reversible activation of PKC, the PKC was identified as an inactive precursor that was activated *in vitro* by Ca^{2+}-dependent proteolysis to a constitutively active fragment termed protein kinase M (PKM). During the persistent phase of long-term potentiation, some of the inhibitory domain of some PKC molecules is proteolyically removed, thus converting it to PKM. PKCζ can also be transcribed from an internal promoter to produce PKM, whose translation in dendrites is unblocked during LTP and whose activity then disables the translational block, an interesting form of molecular memory (Sacktor, 2011). PKC (and PKM) substrates associated with long-term potentiation include NMDA and AMPA receptors.

Protein Tyrosine Kinases Take Part in Cell Growth and Differentiation

Protein kinases that phosphorylate tyrosine residues on key proteins participate in numerous cellular processes and are usually associated with regulation of cell growth and differentiation. Signal transduction by protein tyrosine kinases often includes a cascade of kinases phosphorylating other kinases, eventually activating Ser/Thr kinases, which carry out the intended modification of a cellular process. There are two classes of protein tyrosine kinases. The first is a family of receptor tyrosine kinases that are activated by the binding of extracellular growth factors such as nerve growth factor, epidermal growth factor, insulin, and platelet-derived growth factor. The second family of protein tyrosine kinases, such as c-Src, are soluble kinases that also participate in regulation of cell growth, as well as in neuronal plasticity, but are indirectly activated by extracellular ligands (Lemmon and Schlessinger, 2010).

Why have two sets of amino acids been chosen as targets for phosphorylation? First, the consequences of leaky, or "promiscuous," phosphorylation by a protein Ser/Thr kinase of an unintended target may affect metabolic activity or synaptic function but do not typically initiate irreversible and global functions such as cell growth and differentiation. The consequence of such inappropriate stimulation is seen in the effect of a variety of oncogenes that use altered forms of receptor tyrosine kinases or intermediates in their cascades to subvert normal cell growth. The cellular concentrations of protein Ser/Thr kinases and their targets are much higher than those of protein tyrosine kinases and their substrates. Inadvertent phosphorylation of targets that play critical roles in cell growth is less likely if these targets are regulated at tyrosine residues, which are not well recognized by the numerous protein Ser/Thr kinases. Second, introduction of a phosphotyrosine structure into a protein has a greater regulatory potential than does introduction of a phosphoserine or phosphothreonine. The three phosphorylated amino acids have in common an ability to produce conformational changes due to the extra charge or bulk of the phosphate. For example, in the activation of receptor tyrosine kinases, autophosphorylation displaces an inhibitory domain. In addition, however, the phosphotyrosine and nearby amino acid sequences can be recognized by various signal transduction effectors, such as PLCγ, that contain structural domains that bind to the tyrosine-phosphorylated kinase. The receptor tyrosine kinase thus becomes a platform for concentrating various signaling molecules at specific phosphotyrosine sites in its sequence. These signaling molecules either are activated directly by binding or are activated after having been phosphorylated by the receptor tyrosine kinase. It is easier to bind with the necessary strict specificity to segments of protein around phosphotyrosines, because of the aromatic side chain in Tyr, and this may be an additional reason for use of Tyr as phosphotransferase targets.

Protein Phosphatases Undo What Kinases Create

Protein phosphatases in neuronal signaling are categorized as either phosphoserine–phosphothreonine phosphatases (PSPs) or phosphotyrosine phosphatases

(PTPs) (Hunter, 1995; Mansuy and Shenolikar, 2006). A large emerging class of phosphatase with dual specificity appears to regulate primarily immune and growth signals, often associated with regulation of MAP kinases (Huang and Tan, 2012). Phosphatases catalyze the hydrolysis of the ester bond of the phosphorylated amino acids to release inorganic phosphate and the unphosphorylated protein. They control all of the cellular processes of protein kinases, including neurotransmission, neuronal excitability, gene expression, protein synthesis, neuronal plasticity, and cell growth. They are categorized based on their domain structure, inhibitor sensitivity, and catalytic mechanism (Table 4.1). There are three families of protein serine/threonine phosphatases, designated as phosphoprotein phosphatases (PP-1, -2B (calcineurin), -2A, -4, -6, -5, -7), metal-dependent phosphatases (PP2C), and aspartate-based phosphatases (FCP and SCP). PP-2B (calcineurin) responds directly to a second messenger, Ca^{2+}-activated CaM. The specificity of PP-1 and PP-2A is particularly broad in isolation, but both their protein substrates and cellular localization is refined by interaction with many regulatory and scaffold proteins (Virshup and Shenolikar, 2009). The phosphotyrosine phosphatases constitute a distinct and larger class of phosphatases, including PTPs with dual specificity for both phosphotyrosines and phosphoserine–phosphothreonines. PTPs are either soluble enzymes or membrane proteins with variable extracellular domains that enable regulation by extracellular binding of either soluble or membrane-bound signals.

Structure and Regulation of PP-1 and Calcineurin

PP-1 and calcineurin are the best characterized phosphatases with regard to both structure and regulation. The domain structures of the catalytic subunits of PP-1 and calcineurin are depicted in Fig. 4.14. PP-1 is a protein of 35–38 kDa; most of its sequence forms the catalytic domain; its C terminal is the site of regulatory phosphorylation. The catalytic domains of PP-1, PP-2A, and calcineurin are highly homologous (Price and Mumby, 1999).

PP-1 and PP-2A are normally complexed in cells with specific targeting or regulatory subunits (Price and Mumby, 1999; Hubbard and Cohen, 1993). For example, PP-1 is attached to glycogen particles in liver, myofibrils in muscle, and unidentified targeting subunits in brain. Phosphorylation of the PP-1 targeting

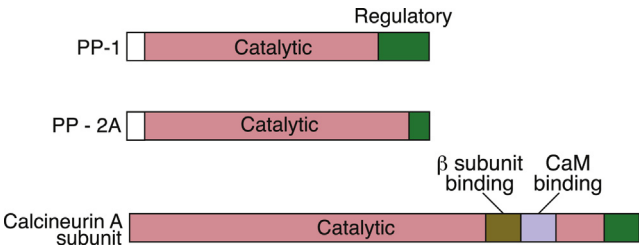

FIGURE 4.14 Domain structure of the catalytic subunits of some Ser/Thr phosphatases. The three major phosphoprotein phosphatases, PP–1, PP–2A, and calcineurin, have homologous catalytic domains (pink) but differ in their regulatory regions (green) and properties.

TABLE 4.1 Categories of Protein Phosphatases[a]

Phosphatase	Characteristic	Other inhibitors
PP-1	Sensitive to phospho-inhibitor 1, phospho-DARPP-32, and inhibitor 2	Weakly sensitive to okadaic acid
PP-2A	Highly abundant	Highly sensitive to okadaic acid
PP-2B (calcineurin)	Ca^{2+}/calmodulin-dependent	FK506, cyclosporin
	CnB regulatory subunit	
PP-2C	Requires Mg^{2+}	EDTA
PP-4/PP-6	Nuclear	Highly sensitive to okadaic acid
PP-5	Nuclear	Mildly sensitive to okadaic acid
PP-7	Nuclear	Mildly sensitive to okadaic acid
FCP/SCP[b]	Aspartic acid-based catalysis	
Receptor PTPs[c]	Plasma membrane	Vanadate, tyrphosphtin, erbstatin
Nonreceptor PTPs	Various cellular compartments	Vanadate, tyrphostin
Dual-specificity PTPs	Nuclear (e.g., cdc25A/B/C and VH family)	Vanadate

[a] *Updated from Hunter (1995).*
[b] *TFIIF-associated component of RNA polymerase II CTD phosphatase / small CTD phosphatase.*
[c] *Protein phosphotyrosine phosphatases.*

subunit in liver releases the catalytic subunit and results in reduced dephosphorylation of substrates near the targeting subunit because the local concentration of PP-1 is reduced as it diffuses away. Targeting of PP-1 also modulates its regulation by natural inhibitors. As PP-1 dissociates from targeting subunits, it becomes susceptible to inhibition by inhibitor 2.

Inhibition of PP-1 by two other inhibitors, inhibitor 1 and its homolog DARPP-32 (dopamine and cAMP-regulated phosphoprotein, M_r 32,000), is conditional on the phosphorylation state of these inhibitors (Hemmings et al., 1984). Inhibitor 1 is broadly distributed in brain whereas DARPP-32 is largely found in the medium spiny neurons in the neostriatum and in their terminals in the globus pallidus and substantia nigra. Both proteins inhibit only after they are phosphorylated by PKA or PKG. PKA also increases the susceptibility of PP-1 to inhibition by stimulating its release from targeting subunits. Because the substrates for PKA and PP-1 overlap somewhat, the rate and extent of phosphorylation of such substrates are enhanced by the ability of PKA to both catalyze their phosphorylation and block their dephosphorylation. Inhibitor 1, DARPP-32, and inhibitor 2 are all selective for PP-1. Highly selective inhibitors capable of penetrating the cell membrane are available for these phosphatases. Okadaic acid, a natural product of marine dinoflagellates, is a tumor promoter, but, unlike phorbol esters, it acts on PP-2A and PP-1, rather than on PKC.

Protein Phosphatase 1. The X-ray structure of the catalytic subunit of PP-1 bound to the toxin microcystin, a cyclic peptide inhibitor, reveals PP-1 to be a compact ellipsoid with hydrophobic and acidic surfaces forming a cleft for binding substrates (Goldberg et al., 1995). PP-1 is a metalloenzyme requiring two metals in the active site that likely take part in electrostatic interactions with the phosphate on substrates that aid in catalyzing the hydrolytic reaction. The phosphate would be positioned at the intersection of two grooves on the surface of the enzyme where binding to amino acid residues on the substrate would occur. Such binding would be blocked when phosphoinhibitor 1 or microcystin LR binds to this surface. The same general structure of the catalytic domain is seen in calcineurin.

Calcineurin (PP-2B). Calcineurin is a Ca^{2+}-calmodulin-dependent phosphatase that is highly enriched in the brain. It is a heterodimer with a 60-kDa A subunit (CnA) that contains an N-terminal catalytic domain and a C-terminal regulatory domain that includes an autoinhibitory segment, a calmodulin-binding domain, and a binding site for the 19-kDa regulatory B subunit (CnB) (Rusnak and Mertz, 2000). CnB is a calmodulin-like Ca^{2+}-binding protein that binds to a hinge region of CnA. Regulation of calcineurin takes place in this region because it controls access of phosphoproteins to the catalytic site. Some activation of calcineurin is attained by binding of Ca^{2+} to CnB. Stronger activation is obtained by the binding of Ca^{2+}-calmodulin.

The Ca^{2+}-calmodulin sensitivity of calcineurin and CaMKII are quite different. Weak or low-frequency stimuli may selectively activate calcineurin whereas strong or high-frequency stimuli activate CaMKII and calcineurin. This difference may play a role in bidirectional control of synaptic strength (depression vs. potentiation) by low- and high-frequency stimulation (Lisman, 1994). (See also discussion of LTP and LTD in Chapter 18.)

Additional regulation may be accorded by interaction of this hinge region with cyclophilin and FK506-binding protein (FKBP), proteins that bind the immunosuppressive agents cyclosporin and FK506, respectively. FKBP is highly abundant in the brain, and its distribution resembles that of calcineurin. Both FK506 and cyclosporin A are membrane permeant and are highly potent and selective inhibitors of calcineurin. They are referred to as immunophilins because their ability to block the essential role of calcineurin in lymphocyte activation makes them effective immunosuppressants. The X-ray structure of calcineurin complexed with FK506 reveals a ternary complex in which FK506 is bound at the interface between FKBP and the regulatory domain of CnA. (Griffith et al., 1995). CnB binds to one surface of an extended regulatory domain of CnA and FKBP−FK506 binds to the opposite surface. The FK506−FKBP complex is wedged between the regulatory domain and the catalytic site and likely inhibits calcineurin by making it difficult for phosphoproteins to have access to the catalytic site. It is unclear whether FKBP and calcineurin interact physiologically via a natural ligand that functions like FK506 to facilitate their interaction.

Protein Kinases, Protein Phosphatases, and Their Substrates are Integrated Networks

Cross-talk between protein kinases and protein phosphatases is key to their ability to integrate inputs into neurons. Such cross-talk is exemplified by the interaction of cAMP and Ca^{2+} signals through PKA and calcineurin, respectively. The medium spiny neurons in the neostriatum receive cortical inputs from glutamatergic neurons that are excitatory and nigral inputs by dopaminergic neurons that inhibit them. A possible signal transduction scheme for this regulation is shown in Fig. 4.15. A more complex scheme is shown in Fig. 4.16 and described in Box 4.2. The key to the regulation is the bidirectional control of DARPP-32

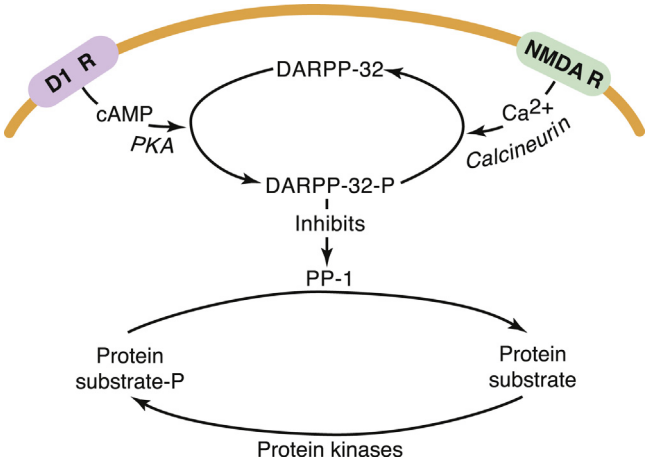

FIGURE 4.15 **Cross-talk between kinases and phosphatases.** The state of phosphorylation of protein substrates is regulated dynamically by protein kinases and phosphatases. In the striatum, for example, dopamine stimulates D1 dopamine receptors (D1 R; purple) to eventually activate PKA, which phosphorylates DARPP−32 converting it into an effective inhibitor of PP−1. This increases the steady-state level of phosphorylation of a hypothetical substrate that exists in a phosphorylated state due to the action by a variety of protein kinases (not shown). This pathway can be countered by NMDA receptor stimulation that increases intracellular Ca^{2+} and activates calcineurin to dephosphorylate and deactive DARPP-32. This causes PP−1 to be deinhibited to dephosphorylate the phosphorylated substrate. *Adapted from Greengard et al. (1999).*

phosphorylation (Greengard et al., 1999; Svenningsson et al., 2004). Glutamate activates calcineurin by increasing intracellular Ca^{2+}, leading to the dephosphorylation and inactivation of phospho-DARPP-32. This releases inhibition of PP-1, which can then dephosphorylate a variety of substrates, including the Na^+, K^+-ATPase, and lead to membrane depolarization. This is countered by dopamine, which stimulates cAMP formation and activation of PKA, which then converts DARPP-32 into its phosphorylated (i.e., PP-1 inhibitory) state. Although PKA and calcineurin are acting in an antagonistic manner, they are not doing it by phosphorylating and dephosphorylating the ATPase. By their actions upstream, at the level of DARPP-32, the regulation of numerous target enzymes (e.g., Ca^{2+} channels and Na^+ channels) in addition to the ATPase can be coordinated.

Studying Cellular Processes Controlled by Phosphorylation−Dephosphorylation Requires a Set of Criteria

Major goals of signal transduction research are to delineate pathways by which signals such as neurotransmitters transduce their signals to modify cellular processes. We do not understand all signaling pathways, their cross-talk with other pathways, or the physiological and pathophysiological roles that they subserve. Such investigation is often the start of a process to identify targets for therapeutic intervention in disease. Cellular and biochemical assays can often identify the entire signaling pathway, from stimulation of receptor, to generation of a second-messenger activation of a kinase or phosphatase, change in the phosphorylation state of the substrate and an ultimate change in its functional state. Signal transduction research utilizes a variety of pharmacological inhibitors or activators of the signaling molecules complemented by genetic approaches that utilize transfection of activated forms of the kinases or phosphatases in question, siRNAs, transgenic animals, and mice with individual signaling components knocked out.

Summary

A morphology of a cell is determined by protein constituents. Its function is regulated by the phosphorylation or dephosphorylation of the proteins. Phosphorylation modifies the function of regulatory proteins subsequent to their genetic expression. The activities of the protein kinases and protein phosphatases are typically regulated by second messengers and extracellular ligands. Kinases and phosphatases integrate and encode stimulation of a large group of cellular receptors. The number of possible effects is almost limitless and enables the tuning of cellular processes over a broad time scale. Potentiation of kinase and phosphatase activity may be key elements in molecular memory and neuronal plasticity. Most of the effects of Ca^{2+} in cells are mediated by calmodulin, which in turn mediates changes in protein phosphorylation−dephosphorylation. The phosphoinositol signaling system is mediated through PKC, which modulates many cellular processes from exocytosis to gene expression. All three classes of enzymes discussed have been described as cognitive kinases because they are capable of sustaining their activated states after their second messenger stimuli have returned to basal levels. PKA has been implicated in learning and memory in *Aplysia* and in hippocampus, where it is involved in long-term potentiation. Protein phosphatases play an equally important role in neuronal signaling by dephosphorylating proteins. Cross-talk between protein kinases and protein phosphatases is key to their ability to integrate inputs into neurons. A major effort of signal transduction research is to delineate the pathways through which the neurotransmitters' signals across the plasma membrane are transmitted to the ultimate cellular components to be modified.

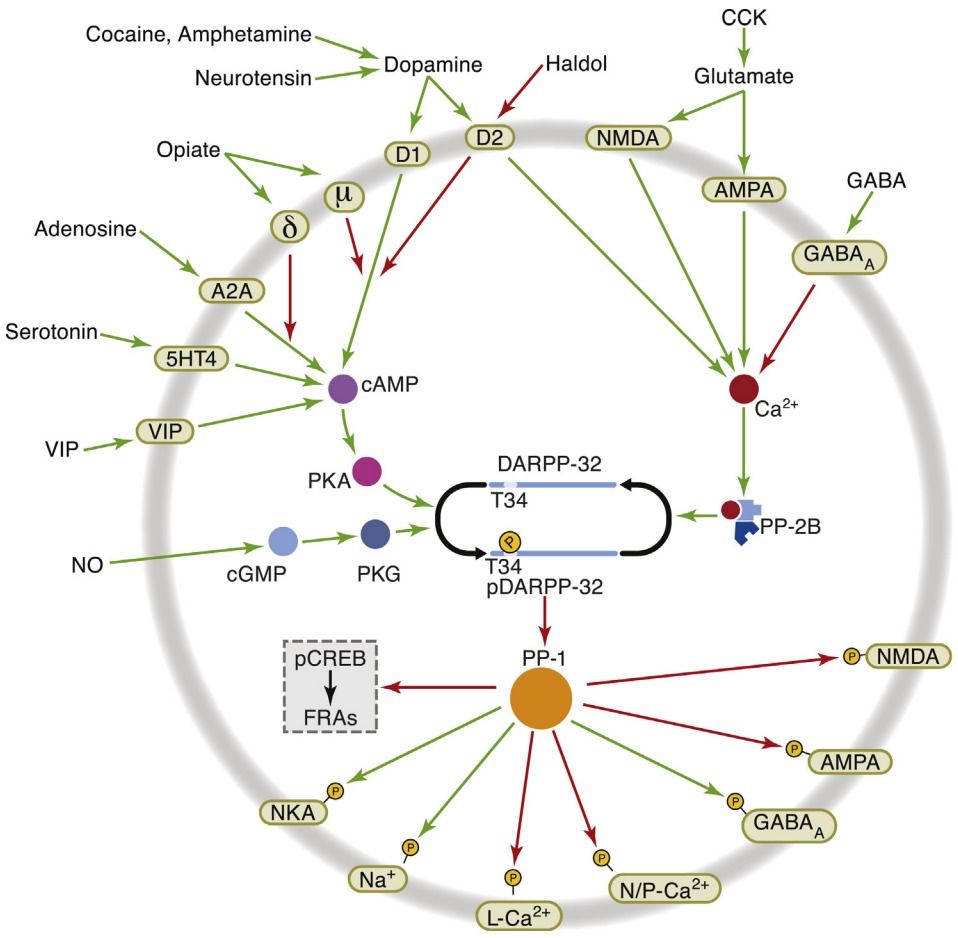

FIGURE 4.16 **Signaling pathways in the neostriatum.** Activation by dopamine of the D_1 subclass of dopamine receptors stimulates the phosphorylation of DARPP-32 at Thr-34. This is achieved through a pathway involving the activation of adenylyl cyclase (increased activity is indicated by green arrows), the formation of cAMP (purple sphere), and the activation of PKA (magenta sphere). Activation by dopamine of the D_2 subclass of dopamine receptors causes the dephosphorylation of DARPP-32 through two synergistic mechanisms: D_2 receptor activation (i) prevents the D_1 receptor-induced increase in cAMP formation (inhibition is indicated by the red arrows), and (ii) raises intracellular calcium (Ca^{2+}; red spheres), which activates a calcium-dependent protein phosphatase, namely, calcineurin, calcium/calmodulin-dependent protein phosphatase 2B (PP-2B). Activated calcineurin dephosphorylates DARPP-32 at Thr-34. Glutamate acts as both a fast-acting and a slow-acting neurotransmitter. Activation by glutamate of AMPA receptors causes a rapid response through the influx of sodium ions, depolarization of the membrane, and firing of an action potential. Slow synaptic transmission, in response to glutamate, results in part from activation of the AMPA and NMDA subclasses of the glutamate receptor, which increases intracellular calcium and the activity of calcineurin, and causes the dephosphorylation of DARPP-32 on Thr-34. All other neurotransmitters that have been shown to act directly to alter the physiology of dopaminoceptive neurons also alter the phosphorylation state of DARPP-32 on Thr-34 through the indicated pathways. Neurotransmitters that act indirectly to affect the physiology of these dopaminoceptive neurons also regulate DARPP-32 phosphorylation; e.g., neurotensin, stimulating the release of dopamine, increases DARPP-32 phosphorylation; conversely, cholecystokinin (CCK), by stimulating the release of glutamate, decreases DARPP-32 phosphorylation. Anti-schizophrenic drugs and drugs of abuse, all of which affect the physiology of these neurons, also regulate the state of phosphorylation of DARPP-32 on Thr-34. For example, the anti-schizophrenic drug haloperidol (Haldol), which blocks the activation by dopamine of the D_2 subclass of dopamine receptors, increases DARPP-32 phosphorylation. Agonists for the μ and δ subclasses of opiate receptors block D_1 and A_{2A} receptor-mediated increases in cAMP, respectively, and block the resultant increases in DARPP-32 phosphorylation. Cocaine and amphetamine, by increasing extracellular dopamine levels, increase DARPP-32 phosphorylation. Marijuana, nicotine, alcohol, and LSD, all of which affect the physiology of the dopaminoceptive neurons, also regulate DARPP-32 phosphorylation. Finally, all drugs of abuse have greatly reduced biological effects in animals with targeted deletion of the DARPP-32 gene. 5HT4, 5-hydroxytryptophan (serotonin) receptor 4; NKA, Na^+, K^+-ATPase; VIP, vasoactive intestinal peptide; L- and N/P-Ca^{2+}, L-type and N/P-type calcium channels. *From Greengard et al. (1999).*

BOX 4.2

INTERACTIONS OF SIGNAL TRANSDUCTION PATHWAYS IN THE BRAIN

An understanding of the signal transduction mechanisms by which neurotransmitters produce their effects on their target neurons and of the mechanisms by which coordination of various signal transduction pathways is achieved represents a major area of research in cellular neurobiology. The dopaminoceptive medium-sized spiny neurons, located in the neostriatum, have been studied in great detail with respect to these mechanisms. Figure 4.16 illustrates a portion of what is now known about interactions of signaling mechanisms in these neurons. Activation by dopamine of D_1 receptors increases cAMP, causing activation of PKA (cAMP-dependent protein kinase) and phosphorylation of DARPP-32 (*d*opamine + c*A*MP-*r*egulated *p*hospho*p*rotein, M_r 32,000) on threonine-34. Conversely, glutamate, acting on NMDA receptors, increases $[Ca^{2+}]_i$, leading to the activation of calcineurin (protein phosphatase 2B, PP-2B) and dephosphorylation of phosphothreonine-34–DARPP-32. Neurotensin, VIP, NO (nitric oxide), and some other neurotransmitters increase the phosphorylation of DARPP-32 through a variety of signaling mechanisms. Dopamine (acting on D_2 receptors), CCK, GABA, and some other neurotransmitters decrease the state of phosphorylation of DARPP-32 through a variety of other signaling mechanisms. CK1 (casein kinase I) and CK2 (casein kinase II) phosphorylate DARPP-32 on residues other than threonine-34, causing it to undergo conformational changes. These changes result in phosphothreonine-34–DARPP-32 becoming a poorer substrate for calcineurin (in the case of CKI) or a better substrate for PKA (in the case of CKII). Antipsychotic drugs such as haloperidol (Haldol) increase the state of phosphorylation of DARPP-32 by blocking the dopamine-induced D_2 receptor-mediated activation of calcineurin.

The physiological consequences of phosphorylation of DARPP-32 on threonine-34 are profound. Thus, DARPP-32 in its threonine-34 phosphorylated, but not dephosphorylated, form acts as a potent inhibitor of PP-1 (protein phosphatase 1). PP-1 is a major serine–threonine protein phosphatase, which controls the state of phosphorylation of a variety of phosphoprotein substrates in the brain. These substrates include Na^+ channels; L-, N-, and P-type Ca^{2+} channels; the electrogenic ion pump Na^+, K^+-ATPase; the NR-1 subclass of glutamate receptors; and probably many more.

In summary, the DARPP-32/PP-1 cascade provides a mechanism by which a large number of neurotransmitters act in a complex, but coordinated, fashion to regulate the state of phosphorylation and activity of a variety of ion channels, ion pumps, and neurotransmitter receptors.

Adapted from Greengard et al. (1999).

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