3 Second-Messenger Systems and Signal Transduction Mechanisms

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CONTENTS

1. INTRODUCTION

1.1. Signal Transduction: From Hormones to Action

Hormones are secreted, reach their target, and bind to a receptor. The interaction of the hormone with the receptor produces an initial signal that, through a series of steps, results in the final hormone action. How does the binding of a hormone to a receptor result in a cellular action? For example, in times of stress, epinephrine is secreted by the adrenal glands, is bound by receptors in skeletal muscle, and results in the hydrolysis of glycogen and the secretion of glucose. Signal transduction is the series of steps and signals that links the receptor binding of epinephrine to the hydrolysis of glycogen. Signal transduction can be simple or complex. There can be only one or two steps between receptor and effect, or multiple steps. Common themes, however, are specificity of action and control: the hormone produces just the desired action and the action can be precisely regulated. The multiple steps that are involved in signal transduction pathway allows for precise regulation, modulation, and a wide dynamic range.

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There are two major mechanisms of signal transduction: transmission of signals by small molecules that diffuse through the cells and transmission of signals by phosphorylation of proteins. The diffusible small molecules that are used for signaling are known as second messengers. Examples of second messengers are cylic adenosine monophosphate (cAMP), calcium (Ca^{2+}), and inositol triphosphate (IP_3). Equally important is the transmission of hormonal signals by phosphorylation. Hormone-induced phosphorylation of proteins is a key way to activate or inactivate protein action. For example, the interaction of epidermal growth factor (EGF) with its receptor stimulates the phosphorylation of a tyrosine residue in the EGF receptor (EGFR). This in turn triggers the phosphorylation of other proteins in sequence, finally resulting in the phosphorylation of a transcription factor and increased gene expression. Enzymes that phosphorylate are called kinases. Balancing kinases are enzymes that remove phosphate groups from proteins; these are called phosphatases. In a typical signal transduction pathway, both second messengers and phosphorylation mechanisms are used. For example, cAMP transmits its message by activating a kinase (camp-dependent protein kinase A, or simply protein kinase A [PKA]).

Some hormones produce effects without a membrane receptor. The best examples of these are the steroid hormones that bind to a cytoplasmic receptor and the receptor then translocates to the nucleus to produce its desired effects. Even these actions, however, are modified by the actions of kinases and phosphatases. Steroid receptors are discussed in detail in Chapter 4.

Nature and evolution are parsimonious. Mechanisms that originally evolved for the regulation of yeast are also used for endocrine signaling in mammals. Similarly, mechanisms used for regulation of embryonic development are also used for endocrine signaling, and mechanisms used for neuronal signaling are also used for endocrine signaling. Thus, fundamental discoveries about the growth of yeast, early embryonic development, regulation of cancerous growth, and neurotransmission in the brain have led to fundamental discoveries of endocrine mechanisms of signal transduction. Similar receptors and signaling pathways underlie signaling by neurotransmitters and by hormones. Growth and differentiation factors trigger cell growth and development by similar mechanisms as do hormones. Thus, signal transduction is a major unifying area among endocrinology, cell biology, developmental biology, oncology, and neuroscience.

1.2. A Brief Overview of Signal Transduction Mechanisms.

One approach to classifying signal transduction mechanisms is as a function of the structure of the hormone receptor. Thus, while both thyroid stimulating hormone (TSH) and growth hormone (GH) are both pituitary hormones, the TSH receptor is a seven-transmembrane G protein–coupled receptor linked to cAMP, and the GH receptor is a single-transmembrane kinase-linked receptor. The fact that both hormones are pituitary hormones tells nothing about the signal transduction mechanism. By contrast, knowledge of the receptor structure involved provides some information as to the potential mechanisms of signal transduction and of the potential mediators involved. Complicating matters, however, hormones can have multiple receptors often with different signal transduction mechanisms. A good example of this is acetylcholine, which has more than a dozen receptors, some of which are seven-transmembrane G protein–coupled receptors and some of which are ligand-gated ion channels.

The major classes of membrane receptors are seven transmembrane, single transmembrane, and four transmembrane. Within each of these classes of receptors, there are multiple signal transduction mechanisms, but certain unifying concepts emerge. The seven-transmembrane receptors are G protein linked, and initial signaling is conducted by the activated G protein subunits. The single-transmembrane receptors convey initial signals via phosphorylation events (sometimes direct, sometimes induced by receptor dimerization), and the four-transmembrane receptors are usually ion channels.

As discussed in Section 2, the seven transmembrane receptors are linked to G proteins. G proteins are composed of three subunits, and binding of the ligand to the receptor G protein complex causes disassociation of the G protein. The disassociated subunit then acts to stimulate or inhibit second-messenger formation. Thus, seven-transmembrane receptors signal through second messengers such as cAMP, IP_3, and/or calcium. Examples of G protein–linked hormones are parathyroid hormone (PTH), thyrotropin-releasing hormone (TRH), TSH, glucagons, and somatostatin. The four-transmembrane receptors are typically ligand-gated ion channels. Binding of the ligand to the receptor opens an ion channel, allowing cellular entry of Na or Ca. Examples of the four-transmembrane receptors are the nicotinic receptors, the AMPA and kainate glutamate receptors, and the serotonin type 3 receptor. The single-transmembrane receptors form the most diverse class of hormone receptors including both single and multisubunit structures. These receptors signal through endogenous enzymatic activity or by activating an associated protein that contains endogenous enzymatic activity.

1.3. Hormone Action: The End Result of Signal Transduction

After hormone binding, there are multiple signaling steps until the hormone actions are achieved. Hormones almost always have multiple actions, so there must be branch points within the signal transduction cascade and the ability to regulate independently these multiple branches. This need for multiple, independently controlled effects is one reason that signal transduction pathways are so diverse and complicated. End effects of the signal transduction cascade fall into three general groups: enzyme activation, membrane effects, and activation of gene transcription. These individual actions are covered in more detail in the specific chapters on hormones, but it is important to understand the general concepts of how signals link to the final action.

The classic example of hormone-induced enzyme activation is epinephrine-induced glycogenolysis in which binding of epinephrine to its receptor (β_2-adrenergic receptor) stimulates formation of cAMP, which activates a kinase (cAMP-dependent protein kinase, PKA). PKA then phosphorylates the enzyme phosphorylase kinase, which, in turn, phosphorylates glycogen

phosphorylase, which is the enzyme that liberates glucose from glycogen. Phosphorylation is the most common mechanism by which hormonally induced signal transduction activates enzymes.

One example of membrane action is cAMP regulation of the cystic fibrosis transmembrane conductance regulator (CFTR), which is a chloride channel that opens in response to PKA-mediated phosphorylation. Another important example of a membrane effect is insulin-induced glucose transport, in which insulin increases glucose transport by inducing a redistribution of the Glut4 glucose transporter from intracellular stores to the membrane.

Hormone-induced gene transcription is mediated by hormone activation of transcription factors or DNA-binding proteins. For steroid hormones and the thyroid hormones, the hormone receptor itself is a DNA-binding protein. How these hormones interact with nuclear receptors to stimulate gene transcription is discussed in Chapter 4. As might be predicted from the preceding paragraphs, membrane-bound receptors stimulate gene transcription through phosphorylation of nuclear binding proteins. Typically, these factors are active only when properly phosphorylated. Transcription factor phosphorylation can be mediated by hormone-activated kinases such as PKA-induced phosphorylation of the cAMP-responsive transcription factor CREB. This is discussed in Section 2.2. GH or prolactin (PRL) stimulates gene transcription by a series of steps leading to phosphorylation of the STAT transcription factors, which then bind and transactivate DNA.

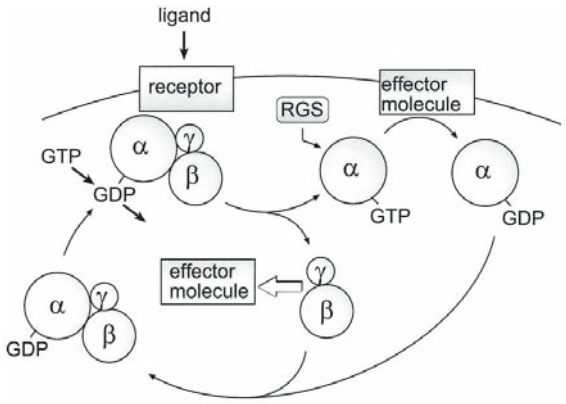

Fig. 1. The G protein cycle. The α-subunit with GDP bound binds to the βγ dimer. The αβγ trimer then binds to the receptor. Binding of ligand to the receptor causes a change in the G protein's conformation such that GDP leaves and GTP is bound. Binding of GTP causes the α-subunit to disassociate from the βγ dimer and assume its active conformation. The activated α-subunit then activates effector molecules. The intrinsic GTPase activity of the α-subunit hydrolyzes the bound GTP to GDP, allowing the α-subunit to reassociate with the βγ dimer. The α-subunit remains activated until the GTP is hydrolyzed. RGS proteins bind to the activated α-subunit to increase the rate at which GTP is hydrolyzed.

2. SIGNALING THROUGH G PROTEIN–LINKED RECEPTORS

2.1. Overview of G Proteins

As described in the previous chapter, the seven-transmembrane receptors signal through G proteins. The G proteins are composed of three subunits: α, β, and γ. The α-subunit is capable of binding and hydrolyzing guanosine 5´ triphosphate (GTP) to guanosine 5´ diphosphate (GDP). As shown in Fig. 1, the trimeric G protein with one molecule of GDP bound to the α-subunit binds to the unliganded receptor. Binding of ligand to the receptor causes a conformational shift such that GDP disassociates from the α-subunit and GTP is bound in its place. The binding of GTP produces a conformational shift in the α-subunit causing its disassociation into a βγ dimer and an activated α-subunit. Signaling is achieved by the activated α-subunit binding to an effector molecule and by the free βγ dimer binding to an effector molecule. Specificity of hormonal signaling is achieved by different α-subunits coupling to different effector molecules. The α-subunit remains activated until the bound GTP is hydrolyzed to GDP. On hydrolysis of GTP to GDP, the α-subunit reassociates with the βγ-subunit and returns to the receptor to continue the cycle. The α-subunit contains intrinsic guanosine 5´ triphosphatase (GTPase) activity (hence, the name G proteins), and how long the α-subunit stays activated is a function of the activity of the GTPase activity of the α-subunit. An important and large family of proteins, the regulators of G protein signaling (RGS) proteins bind to the free α-subunit and greatly increase the rate of GTP hydrolysis to increase the rate at which their ability to signal is terminated.

As shown in Fig. 2, the free βγ dimer can bind to and activate G protein receptor kinases (GRKs) that play a key role in desensitizing G protein–coupled receptors. The activated GRK then phosphorylates the G protein–coupled receptor, which then allows proteins known as β-arrestins to bind to the receptor. The binding of the β-arrestin to the receptor then blocks receptor function both by uncoupling the receptor from the G protein and by triggering internalization of the receptor. Besides the βγ dimers, other signaling molecules can activate GRKs to provide multiple routes to regulate G protein signal transduction.

There are multiple subtypes of the α-, β- and γ-subunits. The subtypes form different families of the G

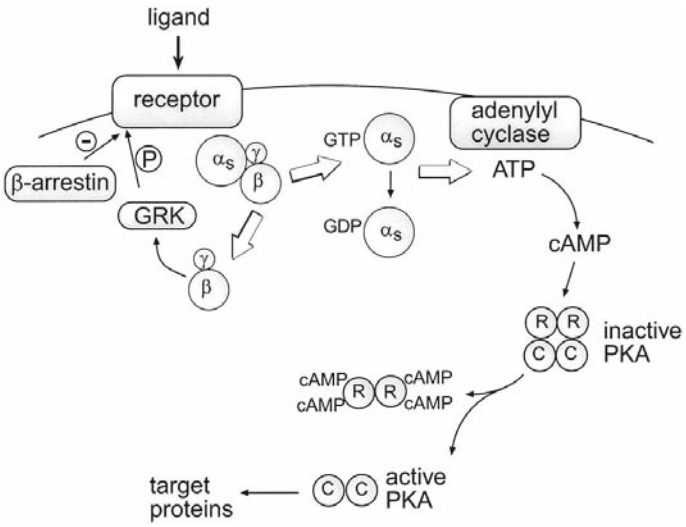

Fig. 2. Signaling by G_s. Binding of ligand to the receptor causes formation of the activated α-subunit of G_s. Activated $G\alpha_s$ then activates adenylyl cyclase. Adenylyl cyclase forms cAMP from adenosine triphosphate. Two molecules of cAMP bind to each regulatory subunit of inactive PKA and cause the regulatory subunits to disassociate from the catalytic subunits. The now-active catalytic subunits can then phosphorylate their target proteins. The free βγ dimer also signals including triggering receptor desensitization by activating GRK proteins to phosphorylate the receptor, which allows the binding of β-arrestin proteins.

proteins. Most important are the subtypes of the α-subunits because they regulate the effector molecules that the G protein activates. The major families of the G proteins are G_S, G_i and G_q. Specificity of hormone action is achieved because only specific G proteins (composed of the proper subunits) will couple to specific hormone receptors and because the free βγ dimer and the activated α-subunit subtypes will couple only to specific effector molecules. The G_s family couples to and increases adenylyl cyclase activity and also opens membrane K^+ channels; the G_i family couples to and inhibits adenylyl cyclase, opens membrane K^+ channels, and closes membrane Ca^{2+} channels; and the G_q family activates phospholipase Cβ (PLCβ) to increase IP_3, diacylglycerol (DAG), and intracellular Ca^{2+}. The signaling of these three families is discussed further in Sections 2.2–2.4.

In addition to the trimeric G proteins discussed above, there is also a class of small G proteins that consist of single subunits, of which Ras, Rho and Rac are important members. These proteins also hydrolyze GTP and play a role in coupling tyrosine kinase receptors to effector molecules, as discussed in Section 3.

2.2. Hormonal Signaling Mediated by G_s

Hormones that signal through G_s to activate adenylate cyclase and increase cAMP represent the first signaling pathway as described by the pioneering work of Sutherland and coworkers in the initial discovery of cAMP. Elucidation of this pathway led to Nobel Prizes for the discovery of cAMP and for the discovery of G proteins. Examples of hormones that signal through this pathway are TSH, luteinizing hormone, follicle-stimulating hormone, adrenocorticotropic hormone, epinephrine, and glucagons, among others. Signaling in this pathway is outlined in Fig. 2. As described in Section 2.1, the binding of hormone to the receptor-G_s complex results in the active α-subunit binding to an effector molecule, in this case adenylate cyclase. Adenylate cyclase is a single-chain membrane glycoprotein with a molecular mass of 115–150 kDa. The molecule itself has two hydrophobic domains, each with six transmembrane segments. Binding of the activated α-subunit of G_s results in catalyzing the formation of cAMP from ATP. Eight different isoforms of adenylate cyclase have been described to date. These isoforms differ in their distribution and regulation by other factors such as calmodulin, βγ subunits, and specificity for α-subunit subtypes. Next cAMP binds to and activates the cAMP-dependent PKA. PKA is a serine/threonine kinase that phosphorylates proteins with the recognition site Arg-Arg-X-(Ser or Thr)-X in which X is usually hydrophobic. PKA is a heterotetramer composed of two regulatory and two catalytic subunits. The regulatory subunits suppress the activity of the catalytic subunits. The binding of cAMP to the regulatory subunits causes their disassociation from the catalytic subunits, allowing PKA to phosphorylate its targets.

There are a number of PKA subtypes, but the key difference reflects the type I regulatory subunit (RI) vs the type II (RII) subunit in which the RI subunit will disassociate from PKA at a lower concentration of cAMP than will the RII subunit. Recent reports have also demonstrated that cAMP can also signal by activating other proteins besides adenylate cyclase.

PKA phosphorylates multiple targets including enzymes, channels, receptors, and transcription factors. Enzymes can be activated or inhibited by the resulting phosphorylation at Ser/Thr residues. An example of regulation of glycogen phosphorylase was discussed in Section 1.3. An example of a PKA-regulated channel is the CFTR chloride channel that requires phosphorylation by PKA for chloride movement. PKA also phosphorylates seven-transmembrane receptors as part of the mechanism of receptor desensitization similar to the function of GRKs.

A key function of cAMP is its ability to stimulate gene transcription. The basic concept is that cAMP activates PKA, which phosphorylates a transcription factor. The transcription factor then stimulates transcription of the target gene. Several classes of cAMP-activated transcription factors have been characterized. These include CREB, CREM, and ATF-1. Probably the most is known about CREB, so it is used here as an example (Fig. 3). CREB is a 341-amino-acid protein with two primary domains, a DNA-binding domain (DBD) and a transactivation domain. The DBD binds to specific DNA sequences in the target genes that are activated by cAMP. When CREB is phosphorylated, it recruits a coactivator protein, CREB-binding protein (CBP). This positions CBP next to the basal transcription complex, allowing interaction with the Pol-II transcription complex to activate transcription. CBP also stimulates gene transcription by a second mechanism by functioning as a histone acetyltransferase. The transfer of acetyl groups to lysine residues of histones is another key mechanism to activate gene transcription. As is almost always the case in signaling cascades, there is important negative regulation of the CREB pathway. A key element of the negative regulation is mediated by phosphorylated-CREB-inducing expression of Icer, a negative regulator of CREB function. Defects in CBP lead to mental retardation in a disease called Rubinstein-Taybi syndrome (RTS), one of the first diseases discovered that is caused by defects in transcription factors.

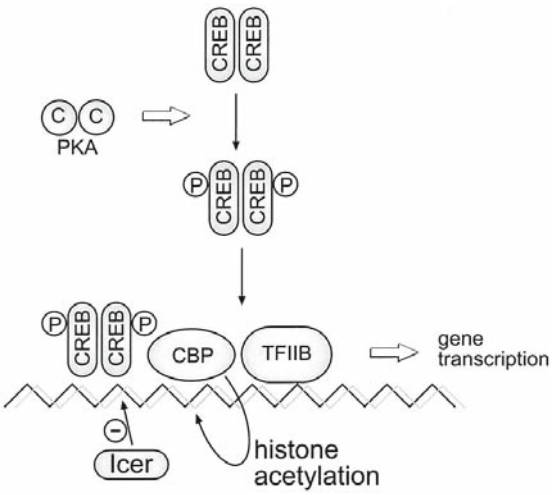

Fig. 3. Role of CREB in regulating gene transcription. PKA phosphorylates CREB on Serine 133. CREB can be phosphorylated while in the cytoplasm (as shown) or while already bound to DNA. The phosphorylation of CREB allows it to bind CBP, which then acts as a transcriptional coactivator by interacting with the pol-II transcription apparatus. CBP also increases gene transcription by acting as a histone acetyltransferase. Icer is an important negative regulator of CREB activity that is induced by CREB.

2.3. Hormonal Signaling Mediated by G_i

Hormonal signaling through seven-transmembrane receptors linked to G_i is similar to that linked to G_s except $G\alpha_i$ inhibits adenylyl cyclase rather than stimulates it, as does $G\alpha_s$. Thus, adenylyl cyclase activity represents a balance between stimulation by $G\alpha_s$ and inhibition by $G\alpha_i$. $G\alpha_i$ also couples to calcium channels (inhibitory) and potassium channels (stimulatory). Receptors that couple to G_i include somatostatin, enkephalin, and the α_2-adrenergic receptor, among others. For G_i signaling, the $\beta\gamma$ dimer also plays key signaling roles by activating potassium channels and inhibiting calcium channels on the cell membrane.

2.4. Hormonal Signaling Mediated by G_q

Hormonal signaling through seven-transmembrane receptors linked to G_q proceeds by activation of PLCβ. Examples of hormones that bind to G_q include TRH, gastrin-releasing peptide, gonadotropin-releasing hormone, angiotensin II, substance P, cholecystokinin, and PTH. Binding of hormone to its receptor leads to formation of active $G\alpha_q$ or $G\alpha_{12}$, which then activates PLC to hydrolyze phosphoinositides (Fig. 4) to form two second messengers, IP_3 and DAG. IP_3 diffuses within the cell to bind to specific receptors on the endoplasmic reticulum (ER). The IP_3 receptor is a calcium channel, and the interaction of IP_3 with its receptor opens the channel and allows calcium to flow from the ER into the cytoplasm, thus increasing free cytosolic calcium levels. The IP_3 receptor is composed of four large subunits (≈310 kDa) that each bind a single molecule of IP_3.

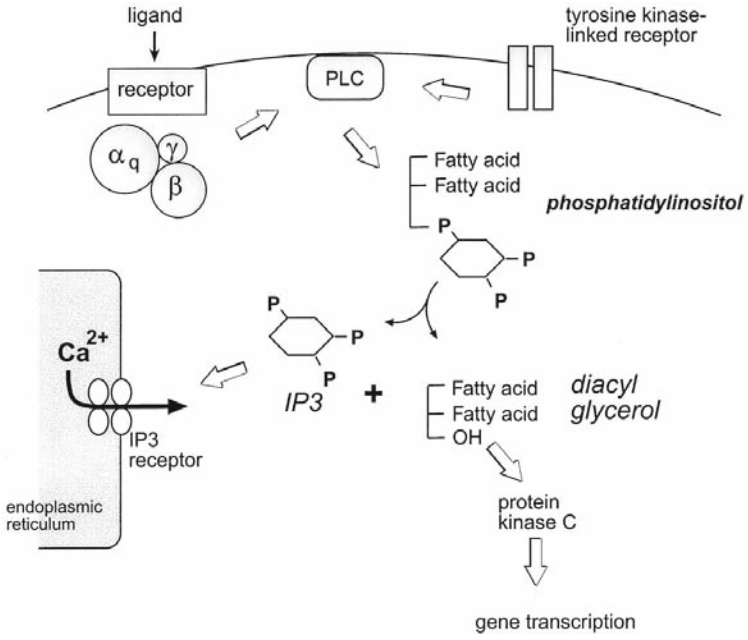

Fig. 4. Signaling by G_q. Activated $G\alpha_q$ activates PLCβ (PLC). PLCβ then hydrolyzes phosphatidylinositol to form two second messengers, DAG and IP_3. The binding of IP_3 to the IP_3 receptor on the ER stimulates calcium efflux from the ER to increase intracellular calcium. DAG activates PKC. PKC can then stimulate transcription by phosphorylation of transcription factors. Tyrosine kinase–linked receptors activate PLCγ to produce DAG and IP_3 as well.

The binding of IP_3 to the subunits opens the channels and also desensitizes the receptor to binding additional IP_3. Thus, IP_3 leads to increased Ca^{2+} which is the next step in signaling. Calcium is returned to the ER by ATP-dependent Ca^{2+} pumps (SERCA). Thapsigargin is a drug that blocks the SERCA, thus resulting in transient high intracellular Ca^{2+} levels, but it also depletes Ca^{2+} levels in the ER, making it a convenient tool to study IP_3-dependent Ca^{2+} release. In excitable cells, a similar mechanism triggers calcium release from internal stores, except here calcium directly triggers additional Ca^{2+} release from the ER via the ryanodine receptor. Depolarization opens voltage-sensitive Ca^{2+} channels on the cell membranes, allowing influx of Ca^{2+}, and this calcium then binds to the ryanodine receptor (very similar to the IP_3 receptor, except the ryanodine receptor is gated by Ca^{2+}) and allows Ca^{2+} efflux from the ER. The ryanodine receptor also allows Ca^{2+} efflux from the sarcoplasmic reticulum in muscle. IP_3, in turn, is rapidly metabolized by specific phosphatases.

Calcium is a major intracellular second messenger, and its levels are tightly regulated by calcium pumps in the ER (SERCA), calcium pumps in the membrane (PMCA), voltage-gated calcium channels, and ligand-gated calcium channels. Resting cell Ca^{2+} is 100 n*M*, far lower than the 2 m*M* levels that occur extracellularly; thus, there is ample room to rapidly increase intracellular Ca^{2+}. Increased intracellular Ca^{2+} signals primarily by binding to proteins and causing a conformational shift that activates their function. Examples include Ca^{2+} binding to troponin in muscle cells to stimulate contraction and Ca^{2+} binding to calmodulin. The Ca^{2+}-calmodulin complex then binds to a variety of kinases. There are two general classes of Ca^{2+}-calmodulin kinases, dedicated, i.e., with only a specific substrate and multifunctional, with many substrates. Examples of dedicated CAM kinases are myosin light chain kinase and phosphorylase kinase. The multifunctional CAM kinases can phosphorylate transcription factors to effect gene transcription. For example, CAM kinase can phosphorylate CREB, which provides a mechanism for cross talk between receptors linked to G_s and G_q. CAM kinases can also phosphorylate other kinases such as mitogen-activated protein kinase (MAPK) or Akt to activate other signaling pathways. In addition, CAM kinases play a key role in mediating signaling by ligand-gated ion channels, as discussed in Section 5.

The other second messenger of the PLC pathway is DAG. The primary action of DAG is to activate PKC, a serine-threonine kinase. PKC modifies enzymatic

activity by phosphorylation of target enzymes, and like PKA, PKC can modify gene transcription by regulating phosphorylation of transcription factors. PKC is activated by the class of compounds known as phorbol esters that were originally described for their ability to promote tumor growth. One phorbol ester that potently stimulates PKC activity is 12-*O*-tetradecanoylphorbol-13-acetate (TPA or PMA). It was initially shown that TPA could activate gene transcription through a DNA sequence element known as the AP-1-binding site. Isolation of the transcription factors that bound to AP-1 led to the isolation of *Jun* and *Fos*, which bind to the AP-1 site as hetero- or homodimers to regulate transcription. Thus, hormones that signal through G_q regulate gene transcription through DAG, which activates PKC, leading to phosphorylation of jun and fos. PKC, like PKA, can also regulate receptor activity by directly phosphorylating ion channels and seven-transmembrane receptors.

3. SIGNALING THROUGH RECEPTORS LINKED TO TYROSINE KINASES OR SERINE/THREONINE KINASES

The second major signaling pathway involves cascades of phosphorylation events. These pathways can be divided into those that commence with a tyrosine phosphorylation event and those that commence with a serine/threonine phosphorylation event. These pathways are similar in that they are a series of protein-binding and/or phosphorylation events. There are two primary mechanism by which the binding of hormone to its receptor causes signal propagation. In the first mechanism, hormone binding triggers receptor autophosphorylation via an intrinsic receptor kinase. Receptor phosphorylation then allows binding of additional proteins that recognize the phosphotyrosines. The EGFR uses this pathway. In the second mechanism, hormone binding triggers a receptor conformational change that stimulates binding of a second protein to the receptor. One important way in which hormone binding to the receptor triggers conformational change is by causing receptor dimerization. Examples of this are the GH and PRL receptors. These are discussed in greater detail in Section 3.2.

3.1. Signaling Through Receptors With Intrinsic Tyrosine Kinase Activity (EGF, Insulin, Insulin-like Growth Factor-1)

Hormones and growth factors that signal through receptors with intrinsic tyrosine kinase activity include the EGFR, the vascular endothelial growth factor receptor, and the insulin receptor. Binding of ligand to the receptor stimulates the receptor's intrinsic tyrosine kinase, resulting in autophosphorylation (i.e., the receptor phosphorylates itself), which then induces binding of the next signaling protein or effector protein. Within this category there are differences depending on receptor structure. Prototype signaling mechanisms are discussed below.

3.1.1. EGFR Signaling

The EGFR is a single-transmembrane receptor that binds EGF as a monomer. EGF binding causes a change in conformation that induces dimerization with a second EGF- EGFR complex. Dimerization of the EGFR complexes activates the EGFR's intrinsic tyrosine kinase, and each receptor in the dimer transphosphorylates the other receptor at multiple tyrosine residues. These phosphotyrosines then serve as docking sites for src homology 2 (SH2) domain proteins. SH2 domains are conserved regions of approx 100 amino acids that serve to target proteins to phosphotyrosines. Depending on the amino acids adjacent to the phosphotyrosine, different SH2 domain proteins will have different affinities for the phosphotyrosine residue. Thus, depending on which tyrosine residues are phosphorylated, and the sequences surrounding those tyrosines, different proteins will dock on the ligand-activated receptor. This provides specificity of effector action and the ability for multiple proteins to dock on a single receptor. The binding of the SH2 domain protein to the receptor propagates signals by a number of mechanisms including 1 bringing an effector molecule to the membrane where it is next to its target molecule, 2 binding that triggers a conformational change that can activate endogenous enzymatic activity in the SH2 proteins (e.g., kinase activity), and 3 binding that can position the SH2 protein so that it can be phosphorylated and activated. The EGFR employs these mechanisms as follows.

As shown in Fig. 5, the binding of EGF to its receptor activates the MAPK pathway, PLCγ, phosphatidylinositol 3-kinase (PI3K), and transcription factors. Many growth factors use pathways similar to EGF, so it is important to consider the multiple pathways of EGF signal transduction. As previously described, Ras is a small G protein with GTPase activity like Rho. When the EGFR is phosphorylated, the SH2 domain protein GRB-2 (growth factor receptor–binding protein-2) binds to the receptor and then binds through its SH3 domain to a guanine nucleotide exchange factor (GEF), which activates RAS by stimulating the exchange of GDP for GTP by RAS. The GEF that binds to the EGFR is known as SOS, or "son of sevenless," because of its homology to the *drosophila* protein) (Fig. 6). This brings SOS close to the membrane and in close proximity to Ras, which is anchored in the membrane. SOS then converts ras-GDP

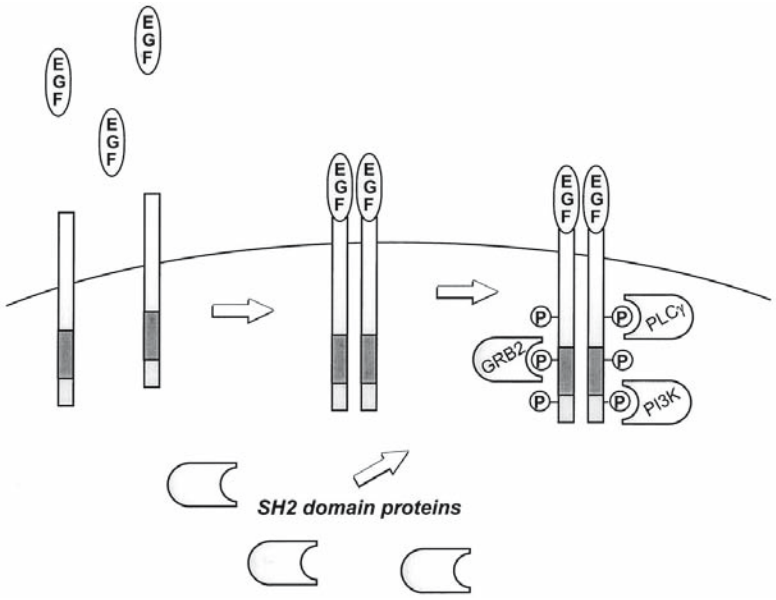

Fig. 5. Signaling by EGFR. Binding of EGF to its receptor causes dimerization of liganded receptors. Receptor dimerization causes receptor autophosphorylation by activating the receptor's intrinsic tyrosine kinase activity (shown in dark gray). SH2 domain proteins such as GRB-2, PLCγ and PI3K then bind to the phosphotyrosine residues. This results in activation of the SH2 domain proteins by either phosphorylation, localization, or both.

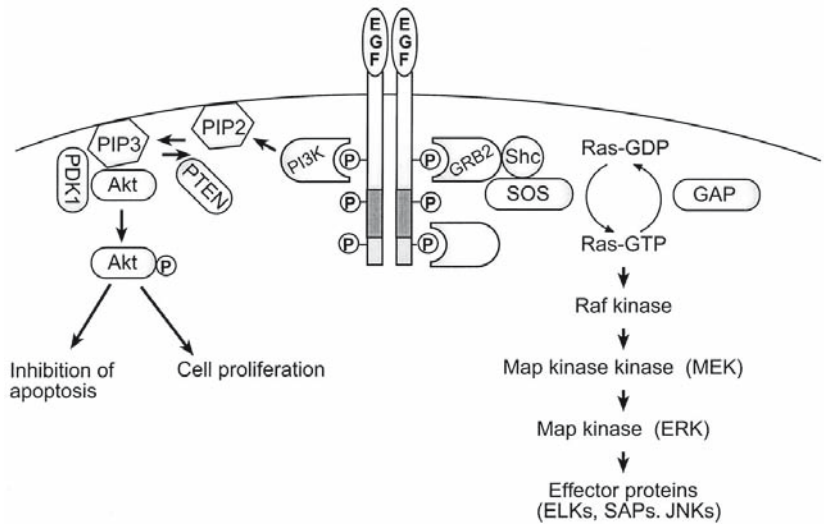

Fig. 6. The MAPK and Akt signaling cascades. Binding of EGF induces phosphorylation of the EGFR, which activates both the MAPK signaling cascade and signaling by Akt. For MAPK activation, the GRB-2-SOS complex binds to the receptor, positioning it near membrane-bound Ras-GDP, which is then activated. The activated Ras GTP activates Raf kinase, which activates MAPK kinase, which activates MAPK which then activates the final effector proteins, many of which are transcription factors. Active Ras-GTP is converted into inactive Ras-GDP by GAP. For Akt signaling, PI3K binds by the SH2 domain, is activated, and converts membrane-bound PIP_2 to PIP_3. PDK1 and Akt bind to PI3K through the Pleckstrin homology domain. This results in phosphorylation to activate Akt, which then triggers cell proliferation by both growth pathways and inhibition of apoptosis. PTEN is a key negative regulator that acts by dephosphorylating Akt.

into the active ras-GTP form. In some systems, SOS does not bind directly to GRB-2, but an intermediate adapter protein, Shc, is recruited, which then binds SOS. Ras-GTP then activates Raf kinase, which activates MAPK kinase, which activates MAPK, which phosphorylates the final effector proteins that regulate growth or cellular metabolism. As always, there is important negative regulation, this time by GTPase-activating proteins

(GAPs) that increase the rate of hydrolysis of GTP bound to RAS to convert RAS to the inactive state. Thus, the GAPs are very similar to the RGS proteins that negatively regulate G protein signaling by increasing the rate of GTP hydrolysis by α-subunits.

There are in fact a number of parallel MAPK pathways with different MAPKs and MAPK kinases. Other MAPK pathways include MEK kinase, which is equivalent to MAPK kinase, and extracellular-regulated kinase (ERK), which is equivalent to MAPK. Transcriptional targets for ERK include the ELK and SAP transcription factors. One important MAPK subtype is Jun kinase, which activates the Jun transcription factors. Specificity of these pathways comes in part from the initial SH2 docking protein that binds to the tyrosine kinase pathways and also from multiple inputs from other proteins. MAPKs are, in turn, rapidly inactivated by phosphatases.

The second major signaling pathway of tyrosine kinase receptors such as the EGFR is through activation of PLCγ. While PLCγ is activated by $G\alpha_q$, PLCγ is an SH2 domain protein. Thus, when EGF stimulates phosphorylation of the EGFR, PLCγ, through its SH2 domains, binds to phosphotyrosines in the EGFR. This serves two purposes: first, it brings PLCγ close to the membrane adjacent to phosphatidyl inositols; and, second, it allows the EGFR to phosphorylate PLCγ. Phosphorylation activates PLCγ resulting in hydrolysis of phosphatidylinositol to IP_3 and DAG. Thus, tyrosine kinase–linked receptors, like G_q-linked receptors, also signal through IP_3 and DAG.

The third major pathway by which the EGFR signals is by activation of other enzymes of which PI3K is one of the most important. PI3K phosphorylates phosphoinositols such as phosphatidylinositol-4,5-bisphosphate (PIP_2) in the 3 position to create phosphatidylinositol-3,4,5-trisphosphate (PIP_3). These phosphoinositols remain membrane bound. The kinase Akt then binds to PIP_3 through a sequence known as the Pleckstrin homology domain. The kinase PDK1 then binds to the Akt and PIP_3 also through the Pleckstrin domain and activates Akt by phosphorylation. Phosphorylated Akt then stimulates cell growth both by inhibiting apoptosis through the BAD pathway and by stimulating growth. Growth stimulation proceeds in part through the phosphorylation of mTOR, leading to activation of protein translation. Negative regulation is provided by the phosphatase PTEN, which dephosphorylates PIP_3. PTEN, because of its ability to counter the growth stimulatory effects of Akt, is an important tumor suppressor. Finally, the EGFR can also directly activate some nuclear transcription factors by phosphorylation.

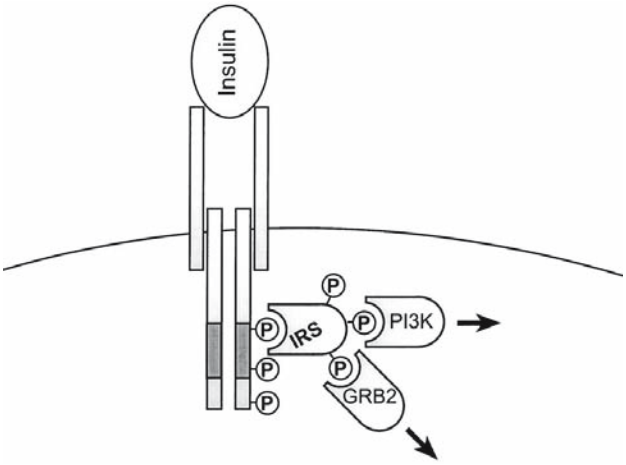

Fig. 7. Signaling by insulin receptor. Binding of insulin to its receptor causes autophosphorylation. This stimulates binding of the IRS protein, which is then phosphorylated by the insulin receptor. SH2 proteins such as GRB-2 and PI3K then bind to the IRS and signal as described for the EGFR. The binding of PI3K to the IRS plays a key role in stimulating glucose entry into cells.

The EGFR has been discussed in depth because it serves as a model for most other tyrosine kinase receptors. The key concept is that ligand binding induces autophosphorylation and SH2 proteins then bind to phosphotyrosines to activate multiple signaling mechanisms. Specificity is achieved in that different SH2 proteins recognize different phosphotyrosines.

3.1.2. Signaling by Insulin and Insulin-like Growth Factor Receptors

The signal transduction mechanism employed by the insulin receptor is a variation of that employed by the EGFR (Fig. 7). Binding of insulin to the insulin receptor (a heterotetramer composed of two α-subunits and two β-subunits), like binding of EGF to its receptor, triggers receptor autophosphorylation. However, the insulin receptor does not signal by directly binding SH2 domain proteins. Rather, ligand-induced receptor autophosphorylation stimulates binding of bridging proteins called insulin receptor substrate (IRS) proteins (IRS1–4). Four IRSs have been described to date, though IRS1 and IRS2 play the key role in insulin signaling. IRSs bind to the insulin receptor and are phosphorylated, and then multiple SH2 proteins bind in turn to the IRSs. Just as EGF-induced signaling depends on which SH2 domain proteins bind to the EGFR, insulin signaling depends on which SH2 proteins bind to the IRS. Examples of proteins that bind to IRSs include GRB-2 and PI3K. GRB-2 then activates the Ras pathway and PI3K activates Akt as discussed above. Akt and PI3K then play key roles in activating glycogen

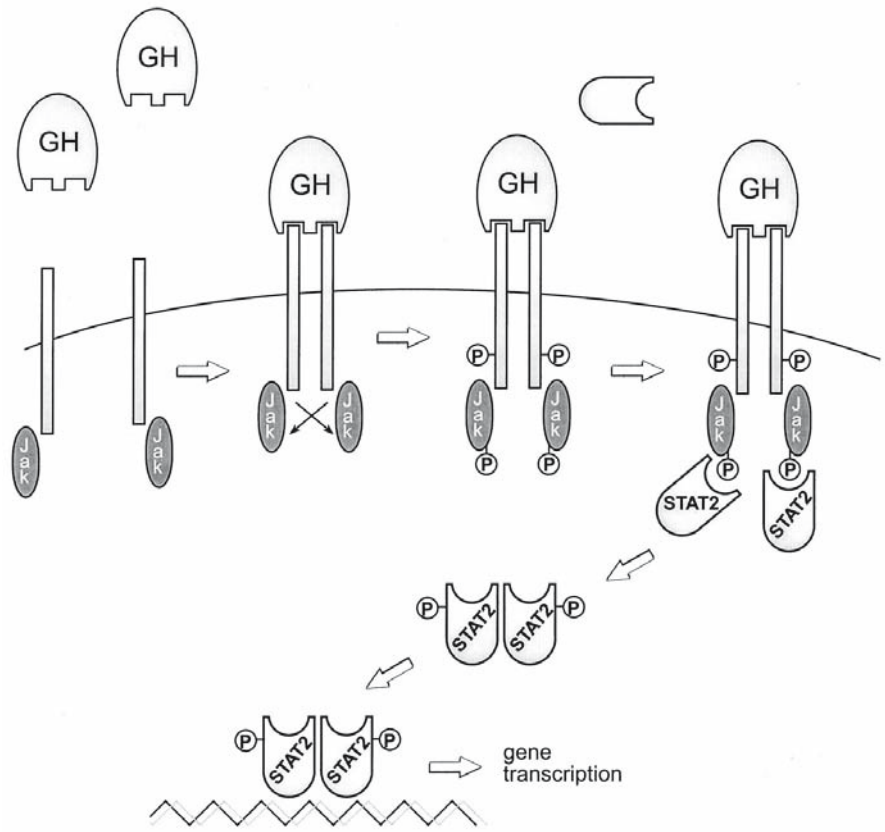

Fig. 8. Signaling by GH receptor (GHR). GH causes receptor dimerization by binding to two receptors. This brings two Jak kinases that are bound to the GHR into close apposition and allows each Jak kinase to phosphorylate the other and the reciprocal GHR (transphosphorylation). Stat proteins then bind through SH2 domains to the Jak kinases and are phosphorylated. The phosphorylated STAT proteins then form homo- or heterodimers, translocate to the nucleus, and stimulate gene transcription.

synthesis and glucose transport into the cell. IRSs do not bind to the insulin receptor via SH2 domains but, rather, appear to utilize Pleckstrin homology domains and phosphotyrosine-binding domains, though the exact details are yet to be determined.

3.2. Signaling Through Receptors That Signal Through Ligand-Induced Binding of Tyrosine Kinases (GH, PRL)

The GH and PRL receptors belong to a large superfamily of receptors that include the cytokine receptors for interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, erythropoietin, granulocyte macrophage colony-stimulating factor, interferon-β (IFN-β), IFN-γ, and CNTF. Many of these receptors are heterodimers consisting of an α-ligand-binding subunit and a β-signaling subunit. However, the GH and PRL receptors have single subunits that contain both the ligand-binding and signaling domains. The receptors in this family lack intrinsic tyrosine kinase activity. Instead, these receptors associate with kinases belonging to the JAK kinase family. Ligand binding to the receptor induces receptor dimerization bringing two JAK kinases in close apposition, which results in activation of the associated JAK kinases by reciprocal phosphorylation (Fig. 8). The JAK kinases then phosphorylate target proteins and signaling commences. The name *JAK kinase* is short for Janus kinase; Janus is the ancient Roman god of gates and doorways who is depicted with two faces, one looking outward, and one looking inward (it has also been claimed that JAK stands for Just Another Kinase). There is a family of JAK kinases and different receptors associate with different kinases. At the present time, four members of the family have been described: Jak1, Jak2, Jak3, and tyk2. The different kinases phosphorylate different targets to achieve signaling specificity. For example, the PRL and GH receptors bind Jak2, the IL-2 and IL-4 receptors bind Jak 1 and Jak3, and the IFN receptors bind tyk2.

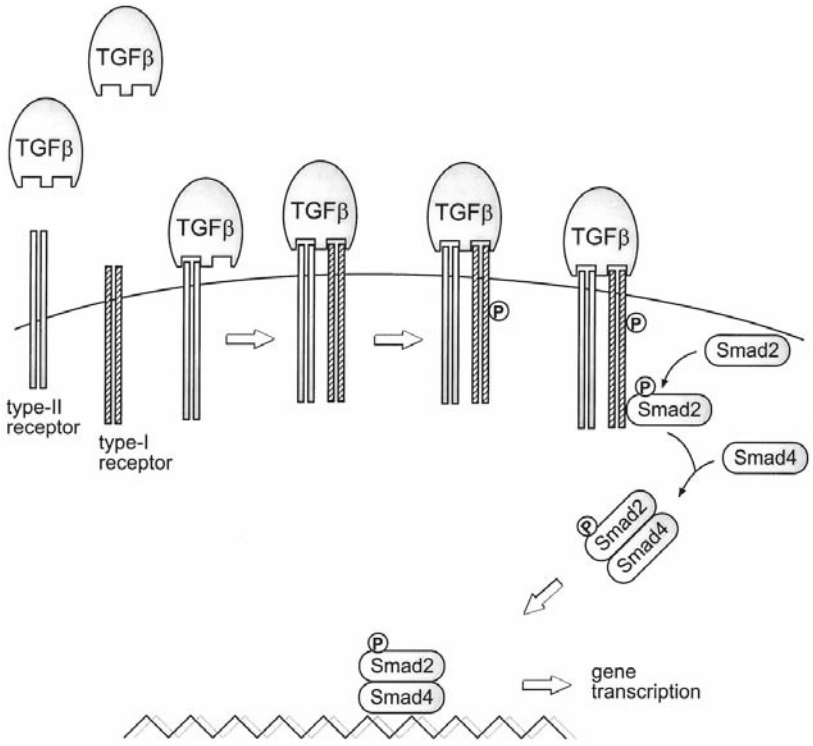

Fig. 9. Signaling by TGF-β receptor. Binding of TGF-β to the type II receptor recruits the type I receptor, which is then phosphorylated. This triggers binding of a Smad protein, which is phosphorylated, dimerizes with a second Smad, and translocates to the nucleus to stimulate transcription.

The activated JAK kinases phosphorylate the signal transduction and activation of transcription (STAT) proteins among others. Seven STAT proteins have been described to date, though there are likely more members of this important gene family. STAT proteins contain an SH2 domain and a single conserved tyrosine residue that is phosphorylated in response to ligand binding. Phosphorylation of STAT releases the STAT from the receptor, and the SH2 domains in the STAT allow them to form as homodimers or as heterodimers with other STATs or with unrelated proteins (Fig. 8). The dimerized STATs can then bind to DNA to stimulate transcription. For example, IFN-α stimulates gene transcription by activation of Stat1 and Stat2, which heterodimerize and bind to DNA. Similarly, CNTF or IL-6 results in binding of Stat1 and Stat3 heterodimers to DNA. A key question remaining to be clarified is, How is exact signal specificity achieved? There are more receptors and ligands than JAK kinases and STATs. Specificity may reside in the time course of activation (reflecting the balance between kinases and phosphatases), which STATs are activated, phosphorylation status of other proteins, and the binding of other transcriptional regulators elsewhere in the gene. Negative regulation results both from STAT-induced transcription of negative regulators and from phosphatases (SHP-1) that dephosphorylate STATs.

3.3. Signaling Through Receptors With Intrinsic Serine/Threonine Kinase Activity (Activin, Inhibin, Transforming Growth Factor-β)

Receptors with intrinsic serine/threonine kinase activity form a large family of receptors. These receptors include the transforming growth factor-β (TGF-β), activin, inhibin, and bone morphogenic proteins. Signaling for TGF-β is best characterized and serves as a model for the signal transduction mechanism of serine/threonine kinase–linked receptors (Fig. 9). TGF-β binds to a type II receptor dimmer, which then recruits a type I receptor dimer. The type II receptor then phosphorylates the type I receptor, which results in the recruitment of Smad proteins, which are the signaling intermediates of the TGF-β receptor. First, Smad2 or Smad3 binds to the TGF-β receptor. Second, the Smad is phosphorylated, disassociates from the receptor, and dimerizes with Smad4. Third the Smad2/3-Smad4 heterodimer translocates to the nucleus and stimulates gene transcription. Negative regulation is achieved by inhibitory

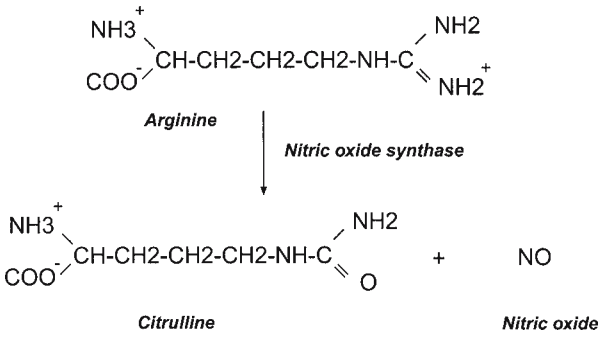

Fig. 10. Formation of NO. NOS and NADPH catalyze the oxidation of arginine to citrulline and NO.

Smads (Smad6, Smad7) which can dimerize with the Smad2 or Smad3 or bind to the TGF-β receptor to prevent signaling.

4. SIGNALING THROUGH NITRIC OXIDE AND THROUGH RECEPTORS LINKED TO GUANYLATE CYCLASE

4.1. Signaling Through Nitric Oxide and Soluble Guanylate Cyclase

Nitric oxide (NO) is one of the more recently characterized signaling molecules. Knowledge of this signaling pathway arose in part from the discovery that NO is the active metabolite of nitroglycerin and other nitrates used for vasodilation. NO is synthesized by oxidation of the amidine nitrogen of arginine through the actions of the enzyme NO synthase (NOS) (Fig. 10). Study of the role of NO has been greatly facilitated by substituted arginine analogs such as L-NAM, which act as potent NOS inhibitors. Because NO has a short half-life, is not stored, and is released immediately on synthesis, NO release reflects regulation of NOS. There are three major forms of NOS: an inducible form present in macrophage, a brain-specific form, and an endothelium-specific form. The brain and endothelial forms are activated by calcium and calcium- calmodulin complexes. The primary signaling mechanism of NO appears to be through cyclic guanosine 5′-monophosphate (cGMP). NO binds specifically to a soluble guanylate cyclase (GC) to stimulate the formation of cGMP. CGMP, in turn, activates ion channels and also activates a cGMP-activated protein kinase (PKG) that can then activate enzymes and signal similarly to PKC and PKA. The soluble GC that acts as the NO receptor is a heterodimer of $Mr = 151{,}000$. However, activation of GC likely does not explain all of NO's actions, and other NO signal transduction mechanisms remain to be determined. NO likely plays an important role in signaling by sensory neurotransmission mediated by neuropeptides such as substance P, vasoactive intestinal peptide, and somatostatin that increase intracellular calcium.

4.2. Hormones That Signal Through Membrane-Bound GC (Natriuretic Peptides)

The action of the atrial natriuretic peptides is mediated by a membrane-bound form of GC. There are three natriuretic peptides: ANP, BNP, and CNP. ANP and BNP bind to GC A (GC-A), and CNP binds to guanylate cyclase B (GC-B). There is a third natriuretic peptide receptor that binds all three peptides. This receptor has been thought to be primarily a clearance receptor, but recent studies suggest that it may also have independent signal transduction properties. GC-A and GC-B are single-transmembrane domain receptors with an extracellular ligand-binding domain, a transmembrane domain, and an intracellular catalytic (GC) domain. Binding of natriuretic peptide to GC-A or GC-B activates the receptors' GC activity, thus stimulating the formation of cGMP. cGMP then signals as discussed above. A third type of membrane-bound GC (GC-C) has also been described in the gastrointestinal tract and kidney. The endogenous ligand of this cyclase may be the small peptide guanylin.

5. SIGNALING THROUGH LIGAND-GATED ION CHANNELS (ACETYLCHOLINE, SEROTONIN)

Although serotonin (5-hydroxytryptamine [5-HT_1]) and acetylcholine (ACh) are most typically thought of as neurotransmitters, they also function as autocrine and paracrine hormones. Serotonin is secreted by pulmonary and gut neuroendocrine cells and ACh by lung airway epithelium. The nicotinic ACh receptors (nAChR) and the serotonin 5-HT_3 receptors are receptors that belong to the family of ligand-gated ion channels. As shown in Fig. 11, binding of the ligand allows calcium or sodium to enter the cell. Depending on the subunit composition, the selectivity for sodium or calcium can vary significantly. Primary signaling is by calcium, which signals by diverse mechanism. Changes in cell potential can open voltage-sensitive calcium channels (VSCCs) to allow more calcium entry to amplify the initial signal. The elevated calcium can then signal through CAM kinase II, which activates the MAPK, Akt pathways, and adenylyl cyclase pathways. Calcium can also activate CAM kinase kinase directly, which further activates Akt. A second important signaling route for calcium is activation of the Ras signaling pathways through mechanisms that involve the EGFR and Pyk2 kinase.

6. CROSS TALK BETWEEN SIGNALING SYSTEMS

As might be imagined, given the complexity and multiplicity of the signaling systems described in this chapter, there is considerable opportunity for cross talk between signal transduction systems. Although signaling systems in this chapter have been discussed as if isolated, it is important to realize that in the cell there is abundant cross activation. For example, multiple hormones can activate the same kinases, and the same kinase can, in turn, phosphorylate targets in more than one signaling pathway. Conversely, one hormone can activate multiple signaling pathways. Thus, signal transduction should not be considered a linear pathway but, rather, a network of activation, and signaling events represent the summation of activation. Equally important is the time course of activation as reflected by the half-life of second messengers and the balance between phosphorylation and dephosphorylation. Cross talk can be at the level of the receptor, second messenger, signaling protein, or transcription factor activation. CREB, e.g., as well as being activated by cAMP, is activated by PKC, Akt, MAPK, and CAM kinase II, making it an important integrator of multiple signaling pathways.

7. DISEASES ASSOCIATED WITH ALTERED SIGNAL TRANSDUCTION

As might be expected, given the diverse mechanisms and multiple effector molecules, there are a number of disease entities associated with signal transduction. A few examples are highlighted here, and more are described elsewhere in this book.

7.1. Oncogenes and Tumor Suppressors

Given the relation between signal transduction and growth, it is not surprising that mutations in signal transduction molecules can lead to unregulated growth and tumorigenesis. Genes that when mutated can cause transformation are called oncogenes (the normal unmutated gene is a protooncogene). Alterations in receptor structure can lead to constitutive activation and constant stimulation of the signaling cascade. An example of this includes the neu oncogene, a point mutation of the EGFR, which leads to rat neuroblastoma and the trk oncogene, a truncation of the nerve growth factor receptor, which occurs in human colon carcinomas. Mutations of the transcription factors jun and fos result in oncogenes carried by avian and murine retroviruses. Similarly, other avian retroviruses carry mutated forms of the tyrosine kinases ras and src. Loss of genes that shut off signaling pathways such as PTEN also results in tumors. This is discussed further in Chapter 19.

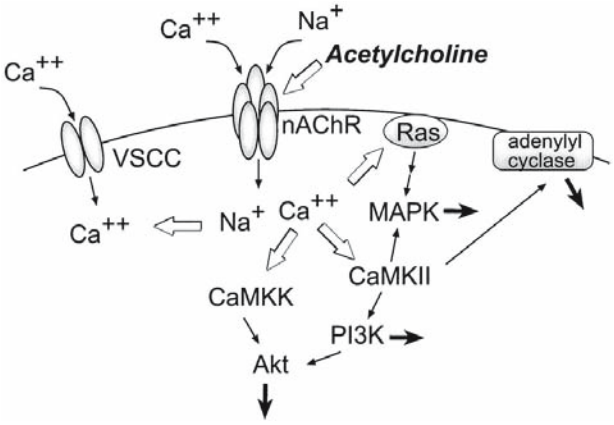

Fig. 11. Signaling by nAChR, a ligand-gated ion channel. The binding of Ach allows calcium or sodium to flow through the channel. Calcium and sodium activate VSCCs and calcium, in turn, can signal through multiple mechanisms. These include activation of CAM kinase II, CAM kinase kinase, Ras, adenylyl cyclase, PI3 kinase, Akt kinase, MAPKs, Pyk2 kinase, and the EGFR.

7.2. Alteration of G Protein Function

7.2.1. Pertussis and Cholera Toxin

Pertussis and cholera toxin are two toxins of major clinical importance that achieve their actions in part by interacting with G protein α-subunits. Cholera toxin causes adenosine 5′-diphosphate ribosylation of the α-subunit of G_s. This has the effect of inhibiting the α-subunit's GTPase activity, thus "locking" the subunit in its active GTP-bound conformation, which increases its ability to activate adenylyl cyclase and results in increased levels of cAMP. Increased levels of cAMP in the intestinal epithelial cells causes fluid secretion throughout the intestinal tract and the massive diarrhea that characterizes cholera. Pertussis toxin causes ADP ribosylation of the α-subunit of G_i. This results in uncoupling of the G protein from the receptor and leads to constitutive activation of adenylyl cyclase and increased levels of cAMP.

7.2.2. Type 1 Pseudohypoparathyroidism

Type I pseudohypoparathyroidism (PHP), also known as Albright's hereditary osteodystrophy (AHO), is a genetic disorder caused by defects in $G\alpha_s$. AHO is characterized by a distinctive phenotype of short stature, round face, obesity, shortened metacarpals, and subcutaneous ossification. In examining kindreds of type I PHP, multiple defects in $G\alpha_s$ have been described. These include point mutations, frame shifts, and splicing mutations that all produce decreased levels of $G\alpha_s$. This results in decreased responsiveness to PTH, which

signals through G_s and, hence, the appearance of apparent hypoparathyroidism. As would be expected, given that G_s mediates signaling for multiple other hormones, patients with PHP exhibit multiple hormone resistance and a variety of cell types have lowered levels of adenylyl cyclase. As well as the hallmark symptoms associated with PTH resistance, patients with AHO frequently exhibit hypothyroidism and hypogonadism. PHP is discussed further in another chapter.

7.3. Alterations in cAMP-Induced Gene Transcription (RTS)

RTS is a well-defined syndrome with facial abnormalities, broad thumbs, broad big toes, and mental retardation. It has recently been discovered that RTS is caused by genetic defects in CBP. Kindreds of RTS have chromosomal break points, microdeletions, or point mutations in the CPB gene. The disease occurs in patients heterozygous for the mutation. Because CPB mediates the ability of cAMP and CREB to stimulate gene transcription, mutations in CPB will interfere with a large number of target genes. How this results in the specific syndrome remains to be determined.

7.4. Alterations in cGMP Signaling (Heat-Stable Enterotoxin)

Some strains of pathogenic bacteria produce a heat-stable enterotoxin. These toxins are a major cause of diarrhea in humans and animals and are a major cause of infant mortality in developing countries. Patients typically present with a watery diarrhea and no fever. These toxins act by binding to the membrane-bound forms of GC to increase cGMP. The increased cGMP appears to cause the diarrhea. There are two forms of heat-stable enterotoxin: STa and STb. STa binds to GC-C which is found in the intestinal mucosa. The exact mechanism by which STa activates GC remains to be determined. Some of the effects of STa may also be mediated by cGMP activation of PKA.

SELECTED READING

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE. Insights into G protein structure, function, and regulation. *Endocr Rev* 2003;24: 765–781.

Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L. VEGF-receptor signal transduction. *Trends Biochem Sci* 2003;28: 488–494.

Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol Rev* 2002;54:527–559.

Kohout TA, Lefkowitz RJ. Regulation of G protein–coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol* 2003;63:9–18.

Kopperud R, Krakstad C, Selheim F, Doskeland SO. cAMP effector mechanisms: novel twists for an 'old' signaling system. *FEBS Lett* 2003;546:121–126.

Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2001;2: 599–609.

McManus KJ, Hendzel MJ. CBP, a transcriptional coactivator and acetyltransferase. *Biochem Cell Biol* 2001;79:253–266.

Petrij F, Giles RH, Dauwerse HG, Saris JJ, Hennekam RC, Masuno M, Tommerup N, van Ommen GJ, Goodman RH, Peters DJ. Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature* 1995;376:348–351.

Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology* 2004;145:2498–2506.

Spiegel AM, Weinstein LS. Inherited diseases involving g proteins and g protein-coupled receptors. *Annu Rev Med* 2004;55:27–39.

Sutherland EW. Studies on the mechanism of hormone action. *Science* 1972;177:401–408.

Ten Dijke P, Goumans MJ, Itoh F, Itoh S. Regulation of cell proliferation by Smad proteins. *J Cell Physiol* 2002;191:1–16.

West AE, Chen WG, Dalva MB, Dolmetsch RE, Kornhauser JM, Shaywitz AJ, Takasu MA, Tao X, Greenberg ME. Calcium regulation of neuronal gene expression. *Proc Natl Acad Sci USA* 2001;98:11,024–11,031.

White MF. IRS proteins and the common path to diabetes. *Am J Physiol Endocrinol Metab* 2002;283:E413–E422.