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COMMENTARY

Introduction to Signal Transduction

A PRIMER FOR UNTANGLING THE WEB OF INTRACELLULAR MESSENGERS

*Kathleen M. Eyster**

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY, UNIVERSITY OF SOUTH DAKOTA, SCHOOL OF MEDICINE, VERMILLION, SD 57069-2390, U.S.A.

ABSTRACT. The field of signal transduction developed from our need to understand the specific mechanisms involved in the transmission of extracellular signals to intracellular actions in target cells. As our comprehension of cellular function has grown, it has become clear that the need to understand signal transduction events has invaded all fields of biological science. Many scientists learned about adenylate cyclase and cyclic AMP when they were students but went on to focus on some other aspect of science; now they find the need to apply studies of signal transduction to their own work. However, the field of signal transduction has progressed so rapidly that from starting with a rudimentary knowledge of adenylate cyclase and cyclic AMP to understanding the field and applying the knowledge to one's own work seems insurmountable. The goal of this commentary is to provide a starting point for those who recognize the need to understand the mechanisms of signal transduction but do not know where or how to begin. BIOCHEM PHARMACOL **55**;12:1927–1938, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. G proteins; protein kinase A; protein kinase C; tyrosine kinase; guanylate cyclase; review

"O, *what a tangled web we weave . . .* "
Sir Walter Scott, Marmion: *A Tale of Flodden Field*

The individual cells of a multicellular organism must communicate with each other in order to coordinate the functions of the various tissues and organ systems of that whole organism. The basis of intercellular communication is typically through the secretion of molecules by one cell, which then bind to receptors and elicit a change in biological action in a second cell type. The mechanism by which binding of a molecule to its receptor at the target cell elicits a change in biological activity of the target cell is called signal transduction; that is, signal transduction is the transmission of an extracellular signal into an intracellular biological effect. Single-celled organisms also receive signals from their environment, which affect their function. They use signal transduction pathways to transduce extracellular signals to intracellular actions similar to those used by multicellular organisms. The field of signal transduction developed from our need to understand the specific activation and inactivation steps involved in the transmission of extracellular signals to intracellular actions in target cells. The field has moved so rapidly in the last few years with the addition of newly described signalling cascades and newly identified interactions between known pathways that current models of signal transduction pathways have begun to appear as a tangled web (Fig. 1). Each discovery of a new pathway or of new pathway components seems to add a new tangle to the threads rather than providing an untangling of the current web. As a result, keeping up with the field of signal transduction has become an increasingly difficult task. For a newcomer to the field, untangling the web of pathway interactions can be especially daunting.

It is not the purpose of this commentary to serve as a comprehensive review of signal transduction. Rather, the purpose here is to provide some untangling of this web of signal transduction pathways by first describing two generic models of signal transduction in order to establish signalling patterns, and then discussing some of the variations and complications within these pathways. Finally, some of the lessons we have learned from studies of signal transduction will be highlighted. Reviews for each signalling pathway and subtopic are referenced so that the reader can obtain more information on topics of specific interest. Essentially, this primer is designed for people who learned about adenylate cyclase and cAMP† in school and have not kept

* Correspondence: Kathleen M. Eyster, Ph.D., Department of Physiology and Pharmacology, University of South Dakota, School of Medicine, 414 E. Clark St., Vermillion, SD 57069-2390. Tel. (605) 677-5159; FAX (605) 677-5787; E-mail: keyster@sundance.usd.edu.

† *Abbreviations:* AKAP 79, A kinase anchoring protein; CREB, cAMP response element binding protein; cAMP, 3′,5′-cyclic adenosine monophosphate; cGMP, 3′,5′-cyclic guanosine monophosphate; EGF, epidermal growth factor; GAP, GTPase activating protein; GRB2/SOS, linker proteins; Gα and Gβγ, subunits of the G protein; IP_3, inositol trisphosphate; JAK, Janus protein kinase; MAPK, MAPKK, and MAPKKK, mitogen-activated protein kinase, mitogen-activated protein kinase kinase, and mitogen-activated protein kinase kinase kinase; PA, phosphatidic acid; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, calcium- and lipid-dependent protein kinase, protein kinase C; PLA_2, PLCβ, PLCγ, and PLD, phospholipases A_2, Cβ, Cγ, and D; PP1, type 1 ser/thr protein phosphatase; PP2A, type 2A ser/thr protein phosphatase; SAPK, stress-activated protein kinase; ser/thr, serine/threonine; SH2, src homology 2; Smad, signalling proteins for receptor ser/thr protein kinases; and STAT, signal transducers and activators of transcription.

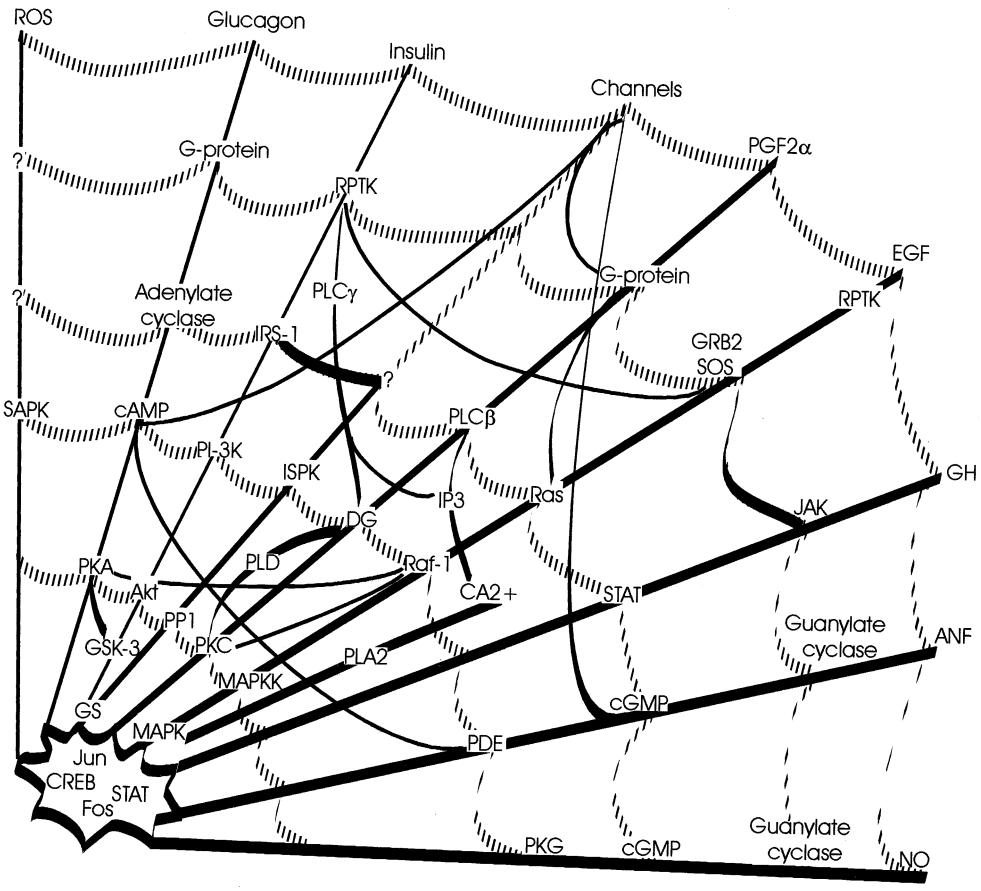

FIG. 1. The tangled web of signal transduction. The outer edge of the web represents the cell membrane; the center of the web represents the cell nucleus. Solid lines depict direct interactions within and among pathways. Abbreviations: Akt, a ser/thr protein kinase; ANF, atrial natriuretic factor; DG, diacylglycerol; EGF, epidermal growth factor; GH, growth hormone; GRB2/SOS, linker proteins in the RPTK pathway; GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; IP_3, inositol trisphosphate; IRS-1, insulin receptor substrate-1; ISPK, insulin-sensitive ser/thr protein kinase; JAK, Janus kinase; Jun, CREB, and Fos, transcription factors; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; NO, nitric oxide; PDE, phosphodiesterase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PI-3K, phosphatidylinositol-3 kinase; PKA, cAMP-dependent protein kinase; PKC, calcium- and lipid-dependent protein kinase, protein kinase C; PKG, cGMP-dependent protein kinase; PLA_2, phospholipase A_2; PLCβ and PLCγ, phospholipase C, β and γ isoforms; PLD, phospholipase D; PP1, type 1 ser/thr protein phosphatase; ROS, reactive oxygen species; RPTK, receptor protein tyrosine kinase; SAPK, stress-activated protein kinase; and STAT, signal transducers and activators of transcription.

up with the field since, but now realize that they cannot ignore signal transduction anymore and do not know where to begin learning again.

GENERIC MODELS OF MEMBRANE RECEPTORS AND THEIR SIGNALLING PATHWAYS

The cell membrane receptor serves as the initiating point for pathways that transduce the signals of water-soluble molecules. There are two generic models of membrane receptors: G-protein-linked receptors and enzyme-linked receptors.

G-Protein-Linked Receptors

In the G-protein-linked receptor model, an extracellular water-soluble molecule, or ligand, binds to a receptor embedded in the cell membrane (Fig. 2). These receptors are called seven-transmembrane pass or serpentine receptors because structurally they pass through the membrane seven times and have both extracellular and intracellular loops [1]. The ligand-binding domain is formed by the extracellular loops of the receptor. Conformational changes elicited by ligand binding to the receptor allow the intracellular loops of the receptor to interact with a linking or transducing protein called a G protein [2]. The G protein consists of three subunits: α, β, and γ. In its inactive state, GDP is bound to the α subunit of this heterotrimeric G-protein complex. Interaction of the ligand–receptor complex with the G protein causes replacement of the GDP on the α subunit with GTP and dissociation of Gα from Gβγ. Gα-GTP then associates with and activates an effector enzyme. As long as it remains associated with Gα-GTP, the effector enzyme will remain active. However,

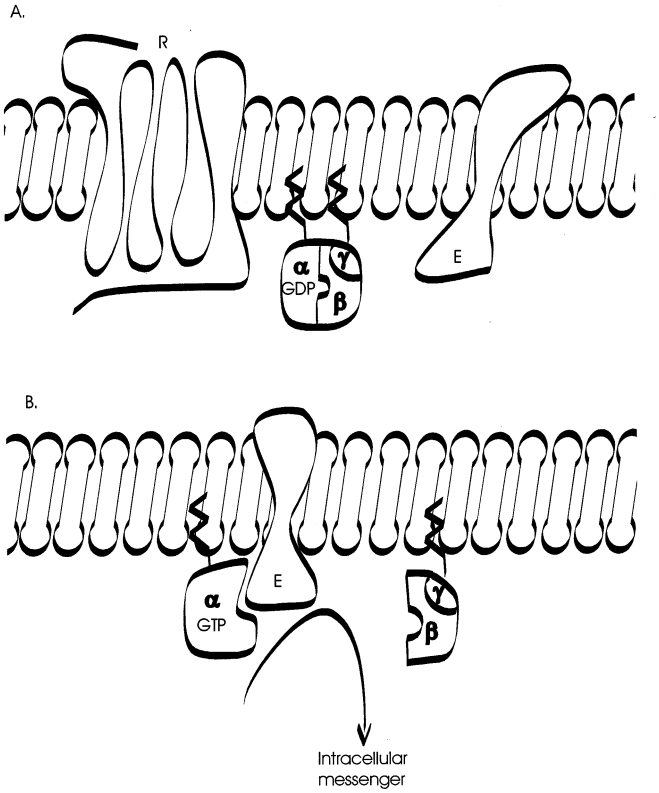

FIG. 2. Model of the G-protein-linked receptor. In the inactive state, GDP is bound to the Gα subunit and the α, β, and γ subunits are associated with each other (panel A). The binding of ligand to receptor (R) results in a change in the interaction of receptor with Gα. GDP is released from Gα and replaced with GTP. The binding of GTP to Gα causes the dissociation of Gα from βγ and allows Gα-GTP to activate an effector enzyme (E) (panel B). The effector enzyme catalyzes the conversion of a precursor molecule to an intracellular messenger. Gα is a GTPase; when it hydrolyzes GTP to GDP, it dissociates from the effector enzyme; inactivating the enzyme, and reassociates with βγ to form the inactive heteromeric complex.

Gα has intrinsic GTPase activity; it will cleave its bound GTP to GDP. The GDP-bound Gα dissociates from the effector enzyme and reassociates with Gβγ; thus, the inactive G-protein complex is reformed, and the activity of the effector enzyme ceases. Down-regulation of the model can occur by a negative feedback mechanism. For example, a downstream messenger in the pathway may activate a kinase that phosphorylates an intracellular region of the receptor to reduce its interaction with the G protein [3].

The adenylate cyclase system is a widespread signalling pathway that uses this generic model [4]. In this pathway, adenylate cyclase is the effector enzyme that is activated by Gα-GTP. Adenylate cyclase catalyzes the conversion of MgATP to cAMP. The cAMP acts as a second messenger; that is, it conveys the message of the extracellular ligand, the first messenger, to the interior of the cell. cAMP activates PKA. In its inactive state, this enzyme consists of two regulatory subunits and two catalytic subunits. Two cAMP molecules bind to each regulatory subunit to allow the release of the catalytic subunits. The free PKA catalytic subunits rapidly phosphorylate cytoplasmic or membrane proteins, thereby changing their activity and causing immediate changes in the biological activity of the cell. PKA may also mediate more chronic effects when the active catalytic subunit enters the nucleus and phosphorylates transcription factors (i.e. CREB), thus affecting the rate of synthesis of specific new proteins [5].

Another pathway that uses the G-protein-coupled mechanism of signalling is the phosphatidylinositol turnover pathway [6]. As described for the generic model (Fig. 2), an extracellular ligand binds to a seven-transmembrane pass receptor and activates a G protein. The dissociated Gα activates a membrane effector enzyme, PLCβ. This enzyme catalyzes the breakdown of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate to IP_3 and diacylglycerol; thus, this pathway results in the release of two intracellular messengers. The water-soluble IP_3 releases calcium from intracellular stores [7], thereby allowing the activation of enzymes and other cellular processes that require calcium. Diacylglycerol, assisted by the membrane lipid, phosphatidylserine, as well as the increased intracellular calcium, activates the calcium- and lipid-dependent PKC [8]. Similar to PKA, PKC has both acute and chronic effects. PKC rapidly phosphorylates cytoplasmic or membrane proteins to achieve acute changes in the biological activity of the cell [8], and also phosphorylates transcription factors to achieve chronic changes in the proteins expressed by the cell [8]. Although both PKC and PKA are serine/threonine protein kinases, the amino acid sequences that they recognize in their target substrates differ [9]. Thus, PKC and PKA recognize and phosphorylate a different but sometimes overlapping series of substrate proteins.

As a side note to this pathway, the terms "trisphosphate" and "bisphosphate" are often misunderstood and misused relative to "triphosphate" and "diphosphate," especially by those not trained as biochemists. With a tris- or bisphosphate, the phosphate groups are attached directly to the parent structure via ester bonds; in the case of inositol 1,4,5-trisphosphate, the parent structure is the inositol ring. With a triphosphate or diphosphate, a chain of phosphate groups in ester linkage is attached to the parent molecule. The structures of inositol 1,4,5-trisphosphate and adenosine triphosphate (Fig. 3) illustrate the biochemical differences between the types of phosphate attachments.

Enzyme-linked Receptors

The enzyme-linked receptor is the second generic model of membrane receptors. In this model, the receptor, membrane-spanning region, and effector enzyme are separate domains of the same protein (Fig. 4A). There are many variations on this generic model. In many cases, ligand binding causes dimerization of these receptors [10]; in other cases, such as the insulin receptor, the receptors are dimerized in the absence of ligand [10]. In both situations, ligand binding to receptor activates the effector enzyme at the intracellular face of the membrane. In another varia-

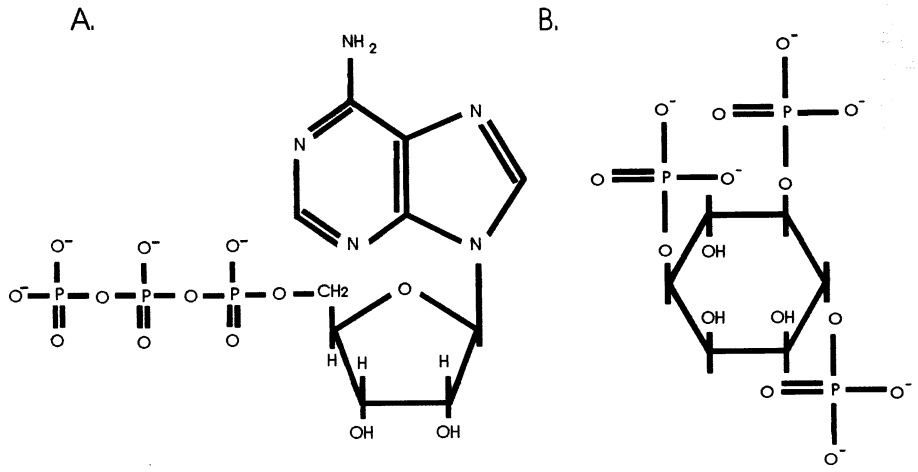

FIG. 3. Structures of adenosine triphosphate (panel A) and inositol 1,4,5-trisphosphate (panel B) to illustrate the difference between a triphosphate and a trisphosphate.

tion, the effector enzyme is a separate protein from the extracellular ligand-binding protein, but the two proteins are closely associated (Fig. 4B). The conformational changes elicited by ligand binding to receptor are conveyed directly to, and activate the effector enzyme without, intervening linker/transducer proteins, such as the G proteins in the model described above.

The most widely studied signalling pathway that uses the enzyme-linked receptor model is the receptor protein tyrosine kinase pathway. Since the effector is immobilized at the intracellular face of the cell membrane, elaborate signalling cascades, such as the MAPK pathway (Fig. 5),

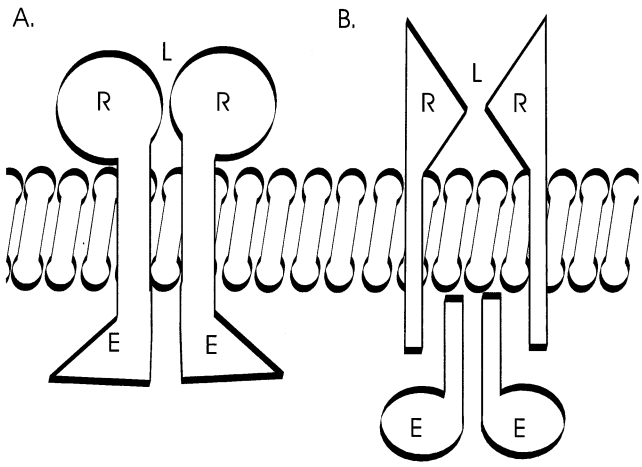

FIG. 4. Model of the enzyme-linked receptor. The molecule consists of an extracellular ligand-binding domain (receptor, R), a transmembrane region, and an effector enzyme (E) at the intracellular face of the membrane (panel A). In some systems, ligand (L) binding causes dimerization of receptor molecules; in other systems, the receptors are dimerized in the inactive, nonligand bound state. Binding of ligand to the extracellular region of the enzyme-linked receptor causes conformational changes in the molecule, which activate the effector enzyme at the intracellular face of the membrane. In one variation of this model (panel B), the ligand-binding domain and effector enzyme are separate proteins, but are closely associated so that the conformational change elicited by ligand binding to the receptor is transmitted to and activates the effector enzyme protein.

have developed to convey messages from the membrane to the nucleus [10, 11]. Ligand binding to the extracellular binding domain activates the effector enzyme, tyrosine kinase, at the intracellular face of the membrane. The receptor protein tyrosine kinase autophosphorylates and also phosphorylates other substrate proteins. The SH2 domain is a specialized protein domain that is designed to interact with tyrosine-phosphorylated residues [12]. Thus, the autophosphorylated receptor serves as a site for binding of proteins containing SH2 domains. This close approximation results in the tyrosine phosphorylation of many of the proteins containing SH2 domains. However, many proteins without SH2 domains also become phosphorylated by receptor tyrosine kinases. In addition, some proteins with SH2 domains are activated by the conformational changes that occur upon binding to phosphorylated tyrosine residues without concomitant phosphorylation of those proteins by the receptor tyrosine kinase.

GRB2 is an example of the class of proteins with SH2 domains that interact with receptor tyrosine kinases without becoming phosphorylated [10]. (In some cell types, another linker protein, Shc, binds to the tyrosine-phosphorylated receptor and becomes tyrosine-phosphorylated. GRB2 then binds the phosphotyrosine of Shc rather than binding directly to the phosphotyrosine of the receptor [10, 13].) The conformational change in GRB2 elicited by the interaction of its SH2 domain with the tyrosine phosphorylated receptor or Shc allows it to interact with another linker protein, SOS (Fig. 5). SOS passes the activation signal on to Ras. Ras is a membrane-associated GTPase with actions analogous to those of the α subunit of the heteromeric G proteins; that is, activation of Ras causes Ras to release GDP and bind GTP. Ras remains active until its inherent GTPase activity hydrolyzes the GTP to GDP. SOS also serves as a GAP. Thus, SOS not only links the ligand activation signal to Ras, but also speeds up the inactivation of Ras by stimulating its GTPase activity [13, 14]. GTP-bound Ras activates Raf-1, a ser/thr protein kinase [13, 15]. Raf-1 is also known as the MAPKKK. Raf-1 phosphorylates and thereby activates the MAPKK.

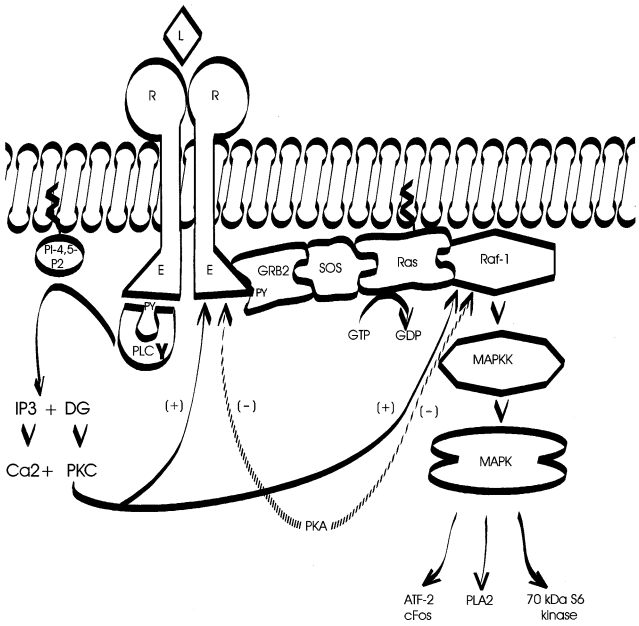

FIG. 5. Receptor protein tyrosine kinase cascade. The largest class of ligands that uses this model is the family of growth factors [10]. Ligand (L) binding to the extracellular binding domain (R) activates the effector enzyme (E), tyrosine kinase, at the intracellular face of the membrane. The receptor protein tyrosine kinase autophosphorylates and also phosphorylates other substrate proteins. The SH2 domain of the linker protein, GRB2, interacts with a tyrosine phosphorylated moiety (PY) of the autophosphorylated receptor. GRB2 then interacts with a second linker protein, SOS, and SOS passes the activation signal to Ras. Ras is activated by the release of GDP and binding of GTP, and remains active until its inherent GTPase activity hydrolyzes the GTP to GDP. GTP-bound Ras activates Raf-1, a ser/thr protein kinase. Raf-1 activates the MAPKK via phosphorylation. MAPKK is a dual-specificity kinase that can phosphorylate both tyrosine and threonine residues. It activates the MAPK by dual phosphorylation. MAPK is a ser/thr protein kinase that can phosphorylate cytoplasmic protein substrates such as PLA_2, other protein kinases such as the 70kDa S6 kinase, and transcription factors such as ATF-2 and cFos. PLCγ is a cytoplasmic enzyme with an SH2 domain that interacts with tyrosine phosphorylated residues (PY) of the receptor protein tyrosine kinase. PLCγ becomes activated by tyrosine phosphorylation and breaks down the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PI-4,5-P2) to IP_3 and diacylglycerol (DG). IP_3 releases Ca^{2+} from intracellular stores. DG, with calcium and membrane phospholipid, activates PKC. PKC can activate the MAPK pathway by a direct interaction with Raf-1 or by actions at the receptor. In contrast, PKA, activated by adenylate cyclase/cAMP, can inhibit the MAPK cascade by interactions with Raf-1 or with the receptor.

MAPKK is a dual-specificity kinase that activates the MAPK by phosphorylation on threonine and tyrosine residues. MAPK is a ser/thr protein kinase that can phosphorylate cytoplasmic protein substrates (such as PLA_2), other kinases (i.e. 70 kDa S6 kinase), and transcription factors (i.e. ATF-2, c-Fos). Thus, this cascade of enzymes transcribes the message of an extracellular ligand to an intracellular change in biological activity.

The JAK-STAT (Janus kinase) signal transduction pathway [16, 17] presents a variation on the enzyme-linked receptor signalling system. In this pathway, the receptor and the effector enzyme are separate but tightly associated proteins (Fig. 4B). Ligand binding to this receptor activates the tyrosine kinase activity of the tightly associated JAK. STAT proteins associate with autophosphorylated JAK by their SH2 domains and become phosphorylated on tyrosine residues. The tyrosine-phosphorylated STAT proteins then dissociate from JAK and form dimers by the interaction of the tyrosine-phosphorylated moiety of each STAT binding to the SH2 domain of the other. STAT dimers translocate to the nucleus where they act as transcription factors. The JAKs can also interact with the linker protein, Shc, and thereby activate the MAPK cascade [13, 18].

The guanylate cyclase signalling pathway also utilizes the generic enzyme-linked receptor model [19]. In this system, ligand binding to the extracellular receptor portion of the molecule initiates the activity of the effector enzyme, guanylate cyclase, at the cytoplasmic face of the membrane. Guanylate cyclase converts GTP to cGMP. The cGMP can then activate such enzymes as cGMP-dependent protein kinase [20] or cGMP-dependent phosphodiesterase [21]. However, the guanylate cyclase signalling system introduces another concept for the transduction of signals. Guanylate cyclase exists as a soluble cytoplasmic form in addition to its enzyme-linked receptor form just described. The soluble form of guanylate cyclase transduces the message of the gaseous signalling molecules, NO [22] and CO [23]. NO and CO are lipid-soluble extracellular messengers that diffuse into the target cells, bind to the heme moiety of the soluble guanylate cyclase, and thereby activate the guanylate cyclase. As with the membrane-bound form of the enzyme, soluble guanylate cyclase converts GTP to cGMP.

Receptor ser/thr kinases also use the generic enzyme-linked receptor model [24]. This system uses Smad proteins [24, 25] to convey signals to the nucleus, but the details of this signal cascade are not yet as well defined as those for the receptor tyrosine kinase/MAPK cascade. For example, it is not known whether there are intervening proteins between the receptor ser/thr kinase and Smad proteins, or whether Smads are, themselves, transcription factors [24].

ADDING TANGLES TO THE WEB

The generic models described above are relatively straightforward. However, the models only serve to establish the basic signalling patterns. In the cell, there are many factors that complicate the relatively straightforward function of these models.

For example, factors that complicate the G-protein-linked model of signal transduction include the presence of multiple G proteins. There are at least 16 known Gα subunits, 5 Gβ subunits, and 11 Gγ subunits [2]. However, there are limits on the combinations of αβγ that can form. Some Gβγ dimers do not form physiologically, and not all α subunits can interact with all Gβγ dimers. This com-

plexity in G proteins provides flexibility; individual receptors can activate different patterns of αβγ complexes, and the various Gα subunits can activate different effector enzymes. Thus, the multiplicity of G proteins provides varying messages to the target cell. Another complicating factor with this model is the activity of Gβγ subunits. In the early models of G-protein activation [26], the Gα subunit was considered the active member of the complex because it directly activated an effector enzyme (i.e. adenylate cyclase or PLCβ); the Gβγ subunits were considered passive binding proteins for the inactive GDP-bound α subunit. It is now known that the Gβγ subunits are not passive; they can also activate effector enzymes [27], but an understanding of the array of their possible actions is not yet complete.

Many ligands have more than one receptor type or subtype, and each receptor may be coupled to a different effector enzyme via various G-protein complexes. Thus, depending on the array of receptors expressed, a given cell may respond to a particular ligand with an increase in adenylate cyclase activity, whereas another cell would respond to the same ligand with an increase in both adenylate cyclase and PLCβ activity. Different Gα subunits have differing intrinsic rates of GTPase activity [28]. In addition, some effector enzymes appear to act as GAPs, which increase the rate of GTPase activity of Gα [13, 26]. Therefore, when a ligand activates two effector enzymes by way of separate Gα proteins, the signal conveyed by the Gα with the greater rate of GTPase activity or associated with a GAP will be quenched more rapidly than the one with the slower rate of GTPase activity or not associated with a GAP.

Another tangle to the generic models is the differential expression of isoforms of enzymes in the pathways. One of the best examples of this is PKC [29]. The conventional isoforms of PKC contain binding domains for calcium, diacylglycerol, and membrane phospholipid such as phosphatidylserine. The new or novel isoforms of PKC lack the calcium binding site, but maintain a requirement for binding diacylglycerol and phosphatidylserine to achieve activation. The atypical isoforms of PKC lack both the calcium and diacylglycerol binding sites. There is clearly a differential distribution of isoforms of PKC across tissue and cell types [30]; however, the physiological relevance of the distribution is not clear.

The MAPK cascade presents a different variation on the concept of "differential expression of isoforms." Analogues of the three kinases, Raf-1, MAPKK, and MAPK, are expressed together as modules; different modules are activated by different ligand/receptor complexes. For example, the conventional MAPK cascade illustrated in Fig. 5 is activated primarily by growth factors such as EGF [10]. The analogous SAPK pathway is activated by cellular stresses such as heat or oxidative stress or by inflammatory cytokines [31].

Subcellular localization provides an additional degree of regulation to many signalling pathways. Anchoring an effector enzyme in a specific location holds it in close proximity to its substrate and provides a stable framework in a signalling cascade. This subcellular localization can also limit the extent of activation of signalling enzymes. Examples of regulation by localization include components of the MAPK cascade that attach to scaffolding proteins, which provide a stable framework between the cell membrane and the nucleus [31], and a targeting protein, which localizes the active type 1 protein ser/thr phosphatase to glycogen particles [32]. Another scaffold protein, AKAP 79, binds to PKA, PKC, and the type 2B protein phosphatase (calcineurin), and thus coordinates their actions in postsynaptic neurons [33]. In addition, many signalling enzymes are localized to cell adhesion complexes and cytoskeletal elements [34].

An even more involved complication is the concept of crosstalk among pathways. When we study signal transduction, we tend to focus on individual pathways, and in the past this has blinded us to the interactions among signalling pathways. In a functioning organism, cells are rarely exposed to a single ligand at any given time. Therefore, cells must be able to integrate incoming messages in order to produce the appropriate biological response to the multiple incoming messages. That is, when a cell is exposed to opposing messengers (i.e. insulin and epinephrine) or to synergizing messages (i.e. glucagon and epinephrine), it must be able to integrate the messages to achieve the appropriate response. Interactions among signalling pathways are a means by which the cell performs the task of integrating the different messages it receives. (Our cells perform this task in an effective and efficient manner, even though our understanding of the process remains in its infancy.)

Crosstalk between G-protein-linked pathways and enzyme-linked receptor pathways is well documented. Growth factors activate the MAPK pathway via receptor protein tyrosine kinases (Fig. 5). PKC, activated by PLCβ via a G-protein-linked pathway, can also activate the MAPK cascade by directly activating Raf-1 [35]. PKA, activated by adenylate cyclase, again by a G-protein-linked receptor, can repress the activation of this pathway by inhibitory interactions with Raf-1 or with the receptor protein tyrosine kinase [35]. In addition, PLCγ is a cytoplasmic protein with an SH2 domain [10]. The SH2 domain of PLCγ interacts with the autophosphorylated receptor protein tyrosine kinase, whereupon PLCγ also becomes tyrosine phosphorylated. The phosphotyrosine-activated PLCγ is then released from the receptor and can catalyze the breakdown of phosphatidylinositol 4,5-bisphosphate to inositol trisphosphate and diacylglycerol, thereby leading to increased levels of intracellular calcium and activation of PKC. Thus, this pathway comes full circle, in that PKC activated by either the G-protein-linked PLCβ or receptor protein tyrosine kinase-activated PLCγ can activate the MAPK cascade. Looking at the picture from a different angle, the receptor protein tyrosine kinase can activate the

MAPK cascade either by way of GRB2/SOS/Ras/Raf-1 or by activation of PKC via PLCγ.

Another illustration of interaction between receptor protein tyrosine kinases and G-protein-coupled receptor pathways is the interaction between the opposing messengers, insulin and glucagon or epinephrine, in the regulation of glycogen synthesis and glycogenolysis (Fig. 6). Glucagon and epinephrine activate adenylate cyclase via a G-protein-coupled receptor. The subsequent increase in cAMP and the resulting activation of PKA stimulates glycogenolysis. Insulin, the opposing hormone, activates a receptor protein tyrosine kinase and a complicated array of downstream messengers (see Fig. 6), which result in glycogen synthesis. Usually we expect nature to take the simplest, most direct path to the regulation of a system, yet the pathway between insulin binding to its receptor and the activation of glycogen synthesis utilizes multiple intervening molecules. We can only speculate on why this is so. Is the convoluted pathway of insulin action related to its physiological function? Insulin is the only hormone that can stimulate storage of all forms of foodstuffs (carbohydrates, proteins, and lipids) for future energy requirements, and it is also mitogenic. In contrast, four hormones (glucagon, epinephrine, cortisol, and growth hormone) can release foodstuffs from their storage forms. Is the convoluted pathway of insulin action designed to provide failsafe mechanisms to prevent inadvertent activation of the pathways to energy storage? Or is it designed to provide a failsafe mechanism to prevent overactivity of insulin mitogenic activity? One might speculate that the former is more likely, since it is believed that insulin mitogenic activity occurs by activation of the MAPK pathway.

Other lipid and lipid-derived mediators in addition to diacylglycerol and inositol 1,4,5-trisphosphate can be generated by multiple pathways. PLD, which releases PA from the membrane lipid phosphatidylcholine, can also be activated by PKC [44]. This could be considered a positive feedback mechanism for maintaining PKC activation since PA hydrolase converts PA to diacylglycerol. PKC activated by either G-protein-linked receptor/PLCβ or receptor protein tyrosine kinase-linked receptor/PLCγ can elicit activation of PLD. Cytosolic PLA_2 releases arachidonic acid (the precursor for prostaglandins, thromboxanes, and leukotrienes) from membrane lipids. PLA_2 is activated by phosphorylation and (for many isoforms) calcium. MAPK has been shown to perform this phosphorylation step, but other protein kinases have been implicated as well [44, 45]. Therefore, ligands that activate the MAPK cascade and ligands that increase intracellular calcium, such as PLC/IP_3/PKC, will result in the activation of cytoplasmic PLA_2. Ligand (primarily cytokine) activation of sphingomyelinase releases another lipid mediator, ceramide, from sphingomyelin [44, 46]. Ceramide then acts as an intracellular messenger.

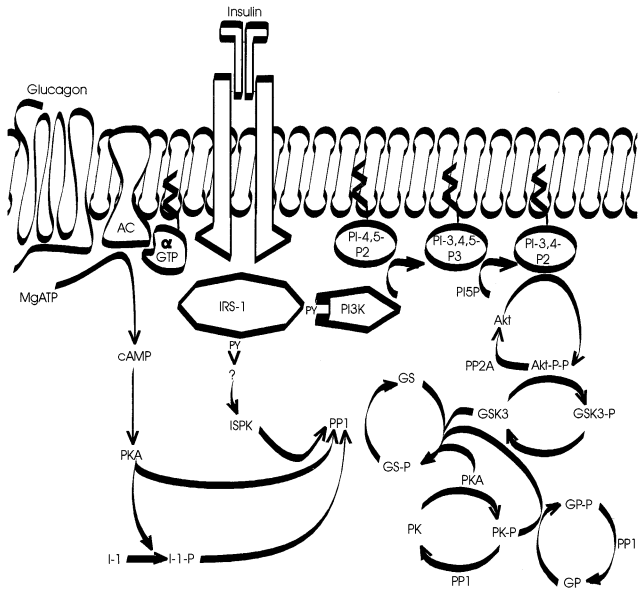

FIG. 6. Regulation of glycogen synthesis by insulin and glucagon. Glucagon binds to a seven-transmembrane pass receptor, activates a G-protein (shown as αGTP), and thereby stimulates adenylate cyclase (AC) activity. Adenylate cyclase converts MgATP to cAMP, and cAMP activates PKA. PKA phosphorylates and thereby activates phosphorylase kinase (PK); phosphorylase kinase activates glycogen phosphorylase (GP) via phosphorylation. Glycogen phosphorylase stimulates glycogenolysis. PKA also inhibits the opposing pathway of glycogen synthesis. It inhibits glycogen synthase and the type 1 ser/thr protein phosphatase (PP1) directly by phosphorylation, and indirectly by phosphorylating and activating inhibitor 1 (I-1, an endogenous inhibitor of PP1). Phosphorylation of PP1 by PKA causes release of PP1 from the targeting protein that localizes it to glycogen particles. The released PP1 becomes a target for I-1 [36]. Insulin binds a receptor protein tyrosine kinase. The receptor autophosphorylates and also phosphorylates insulin receptor substrate-1 (IRS-1) [37]. The resulting tyrosine-phosphorylated sites in IRS-1 (PY) serve as docking sites for proteins with SH2 domains. The binding of phosphatidylinositol 3-kinase (PI3K) to IRS-1 by its SH2 domain activates this enzyme [38]. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PI-4,5-P2) on the 3 position to yield phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P3). Phosphatidylinositol 5-phosphatase (PI5P) then cleaves the phosphate from the 5 position to yield phosphatidylinositol 3,4-bisphosphate (PI-3,4-P2) [39]. The phosphatidylinositol 3,4-bisphosphate activates a ser/thr protein kinase called Akt [39, 40]. The mechanism whereby Akt becomes activated is not yet clear. The phosphatidylinositol 3,4-bisphosphate may localize Akt to a membrane kinase that phosphorylates Akt [39], or the association of Akt with phosphatidylinositol 3,4-bisphosphate may allow Akt to dimerize and autophosphorylate [41]. Activated Akt inhibits glycogen synthase kinase-3 (GSK-3) by phosphorylation [40]. GSK-3, in conjunction with PKA and phosphorylase kinase, inactivates glycogen synthase (GS) via phosphorylation [42]. Therefore, inactivation of GSK-3 by Akt will reduce the inactivation of glycogen synthase and allow glycogen synthesis to proceed. In addition, an insulin-sensitive ser/thr protein kinase (ISPK) activates the type 1 protein phosphatase (PP1). (It is not yet known whether the kinase that activates PP1 is Akt or a different insulin-sensitive protein kinase.) PP1 activates glycogen synthase by removing the phosphate groups added by GSK-3. PP1 also inactivates phosphorylase kinase and glycogen phosphorylase by dephosphorylation [43], thereby preventing glycogenolysis.

MORE TANGLES

The above discussion of signal transduction models illustrates several points. First, each of these major signalling pathways utilizes one or more protein kinases. The role of protein kinases is to phosphorylate substrate proteins and thereby modify the activity of those target proteins. Second, when we consider phosphorylation reactions, we typically think of the change in activity of the target protein as an activation of that protein; that is, as an "on" reaction. Third, our discussion of generic signal transduction models left out the class of enzymes that remove phosphate groups from substrate proteins; that is, the protein phosphatases.

The notion that phosphorylation reactions are "on" reactions that activate target proteins has been perpetuated by the emphasis on the role of protein kinases in signal transduction events. However, it also ignores one of the first lessons we all learned in our basic biochemistry courses: activation of glycogen synthesis utilizes a series of *dephosphorylation* reactions. Energy stored in the form of glycogen, driven by the extracellular ligand insulin, is clearly a protein phosphatase-regulated process (Fig. 6). In this important cellular function, phosphorylation is an "off" reaction and dephosphorylation is an "on" reaction. Another example in which phosphorylation is an "off" reaction and dephosphorylation an "on" reaction utilizes the cytoplasmic tyrosine protein kinase src [47]. Src contains an internal SH2 domain. Tyrosine phosphorylation of src by a tyrosine protein kinase allows an intramolecular interaction of the phosphorylated tyrosine residue with the internal SH2 domain, which inactivates the tyrosine kinase activity of src. Dephosphorylation of src by a tyrosine protein phosphatase reactivates the src kinase activity [47].

Until recently, the potential of protein phosphatases to act as intracellular signals responding to extracellular ligands was largely ignored. Phosphatases were considered to be unregulated housekeeping enzymes that removed phosphate groups from proteins in a rather indiscriminate manner. However, extensive documentation illustrates that not only are protein phosphatases tightly regulated in response to extracellular ligands, but also that they exhibit substrate specificity [48].

A contributing factor to the concept that phosphatases were unregulated enzymes was the early observation that phosphatases were frequently active in tissue preparations, regardless of the state of activation of the tissue (i.e. absence versus presence of extracellular ligands). It is now known that many phosphatases can be inhibited by phosphorylation, but they have the ability to reactivate themselves by autodephosphorylation [49]. This process can occur during tissue processing with the result that the phosphatases appear to be constitutively active. If the phosphatases are inhibited during tissue processing, and then reactivated at the time of enzyme assay, the measured enzyme activity more accurately reflects the enzyme activity of the endogenous state of the tissue [50–52].

Protein phosphatases are divided into two broad categories based on their ability to dephosphorylate serine or threonine residues as opposed to tyrosine residues. The ser/thr protein phosphatases are traditionally divided into four families (types 1, 2A, 2B, and 2C), but there has been recent expansion of the groups and the terminology as new phosphatases have been identified [53]. These families offer several excellent examples of tight control of phosphatase activity [54, 55]. PP1, the type 1 ser/thr protein phosphatase, can be regulated by direct phosphorylation, targeting of the enzyme to specific subcellular locations, and by interaction with endogenous inhibitory proteins [54, 55]. In the example of glycogen synthesis (Fig. 6), PP1 is the protein phosphatase responsible for the dephosphorylation reactions that activate glycogen synthesis. PP1 is associated with (targeted to) glycogen particles and is activated by phosphorylation by an insulin-sensitive protein kinase [32]. A second phosphorylation of PP1 by PKA, activated by epinephrine or glucagen, causes release of PP1 from the glycogen particles and partial inactivation of the enzyme. PKA also phosphorylates, and thereby activates, inhibitor 1, which is an endogenous inhibitor of PP1 [54]. The release of PP1 into the cytoplasm allows it to interact with inhibitor 1 and finalizes the inhibition of PP1. Dephosphorylation of PP1 and of inhibitor 1 are necessary to reactivate this protein phosphatase. The activities of the other ser/thr protein phosphatases are also carefully regulated. PP2A, the type 2A protein phosphatase, can be inhibited by phosphorylation or methylation [49, 54–56] of the catalytic subunit, or by the presence of regulatory subunits [49, 54, 55]. The type 2B protein phosphatase (calcineurin) is the calcium/calmodulin-dependent protein phosphatase [54, 55] and is thus activated by binding to calcium/calmodulin.

The examples of extracellular ligand regulation of protein phosphatases described above are all downstream from activation or inhibition. However, direct regulation of a protein tyrosine phosphatase by a G-protein-linked receptor has been described [57]. In addition, a class of enzyme-linked receptors with protein tyrosine phosphatase as the effector enzyme (Fig. 4A) is involved in cell–cell signalling [58].

A protein kinase recognizes its substrates by the 2–4 amino acids on either side of the serine, threonine, or tyrosine residue that it will be phosphorylate. Peptides containing the appropriate sequences are readily phosphorylated by kinases, and exclusive peptides specific for individual kinases have been designed and are commercially available. The terminal (gamma) phosphate group transferred from ATP to substrate by a protein kinase includes three oxygens in addition to the phosphorus (PO_3^{2-}) (see Fig. 3). The conformation of the substrate protein must change to accommodate the new prosthetic group with its attendant negative charges. Therefore, the phosphorylated protein, which is the substrate for a protein phosphatase, is not simply the equivalent of the kinase substrate with the attachment of a phosphorus. Crystal structures of proteins in their phosphorylated state compared with their dephos-

phorylated state serve to further illustrate this point [59]. Many protein phosphatases are unable to recognize phosphorylated peptides as substrates, even though those peptides may have been derived from the phosphoprotein sequences known to be substrates for the phosphatase [60]. To recognize their substrates, these protein phosphatases require the three-dimensional structure of the substrate protein surrounding the phosphate group that is to be removed, rather than just the amino acid sequence on either side of the phosphorylated amino acid. Thus, although *in vitro* studies show cross-reactivity of protein phosphatases with their substrates, the potential exists for tighter control of substrate specificity for protein phosphatases than exists for protein kinases.

In our zeal to document the regulation of protein kinases in signalling pathways, we have neglected other forms of signalling in addition to protein phosphatases. For example, cyclic nucleotides (i.e. cGMP and cAMP) can directly open ion channels in the cell membrane, thereby causing changes in ion fluxes [61]. In fact, one of the most fascinating examples adding unique twists to the G-protein-coupled model of signal transduction to achieve regulation of cellular function is the transduction of light in rod cells of the retina [62]. Signal transduction pathways typically cause an *increase* in the concentration or activity of intracellular messengers. In the rod cells, activation of the signalling pathway results in a *reduction* in concentration of the signalling molecule. Rod cells are responsible for black/white vision in low-light situations. In the absence of light, cGMP holds sodium channels open in the cell membrane of the rod outer segment; these channels also admit calcium and magnesium to the cell. In this system, the seven-transmembrane pass receptor is rhodopsin, transducin is the G protein, and cGMP phosphodiesterase is the effector enzyme. Rhodopsin is covalently attached to a light-sensitive molecule, 11-*cis* retinal. The ligand for this signalling pathway is a photon of light. The 11-*cis* retinal absorbs the photon of light ($h\nu$) and isomerizes to all-*trans* retinal. Isomerization of the retinal activates the rhodopsin so that it interacts with the G protein, transducin. Like other G proteins, activation of transducin causes exchange of GTP for GDP on the $G\alpha$ subunit with subsequent dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer. The effector enzyme activated by $G\alpha$-GTP is a cGMP PDE. cGMP PDE breaks down cGMP. As the concentration of cGMP decreases, the sodium channels in the cell membrane close. Thus, in this system, activation of the signalling pathway causes a reduction in the concentration of the signal, cGMP, and the change in biological function of the cell occurs in response to this decrease in cGMP.

The example of visual signalling illustrates another point that is often neglected in signal transduction; that is, ion channels can be opened and closed by intracellular messengers. Therefore, channels can be considered effectors for signal transduction pathways. Regulation of channels by cGMP is not confined to rod cells of the retina, but occurs in other cell types as well [63]. cAMP can also directly regulate channels [61]. Some ion channels are regulated by phosphorylation/dephosphorylation [63, 64], and $G\beta\gamma$ dimers have also been implicated in channel regulation [65].

ALL WEBS LEAD TO THE NUCLEUS

Often neglected in a discussion of signal transduction events are the lipid-soluble signalling molecules such as the steroid hormones (progesterone, estrogen, testosterone, cortisol, and aldosterone), 1,25-dihydroxyvitamin D_3, and the thyroid hormones. These factors do not require transmembrane signal transduction mechanisms because they have the ability to cross the cell membrane. They bind to intracellular receptors, and the lipid-soluble ligand/receptor complex acts as a transcription factor in the nucleus [66]. In addition to these genomic actions, emerging evidence indicates that lipid-soluble hormones have acute, non-genomic effects [67, 68]. These data suggest that lipid-soluble hormones can bind to membrane receptors and activate signalling pathways of the same type as the generic membrane models of signal transduction; that is, receptor tyrosine kinase activation [67, 69], increases in calcium fluxes [67, 68], and increases in chloride flux [67–69], as well as other acute effects, have been reported. References to acute effects of lipid-soluble hormones, which occur too rapidly to be mediated by genomic effects, have been well-documented for many years [67, 68]; however, these effects and their mechanisms of action were largely ignored because of the compelling descriptions of the genomic mechanism of action for these lipid-soluble ligands. It seemed superfluous to invoke another mechanism of action for these hormones. However, it now seems clear that lipid-soluble hormones can cause rapid effects by activation of membrane receptor signalling pathways, as well as causing chronic genomic effects by binding to intracellular receptors and acting as transcription factors, as usually described for these hormones. Similarly, the signalling pathways described in the generic models cause both rapid changes in the activity of cytoplasmic proteins, frequently via phosphorylation or dephosphorylation events, and also genomic changes by phosphorylation of transcription factors. Thus, all pathways, whether initiated by factors that cannot enter the cell or by factors that can diffuse through the cell membrane, have the ability to effect rapid changes in the activity of cytoplasmic proteins, as well as chronic changes by effects on DNA transcription.

SUMMARY AND CONCLUSIONS

The field of signal transduction emerged in the 1950s when Sutherland and his coworkers identified cAMP as an intracellular messenger elicited by the extracellular application of epinephrine or glucagon to liver cells [70], and when Fischer, Krebs, and co-workers described the importance of reversible phosphorylation to cell function [71]. There are many lessons we can learn about science, in

general, by an examination of the development of this field. One lesson is that sometimes we do not find what we are looking for in our studies because our expectations are misdirected. One example of this phenomenon in the field of signal transduction is the study of cGMP. cGMP was described within a few years of the description of cAMP [72]. Since cAMP mediated the effects of epinephrine and glucagon, and the actions of insulin opposed the actions of those hormones, at first it was expected that cGMP would mediate the effects of insulin (the so-called yin-yang theory). Further, it was expected that guanylate cyclase would behave similarly to adenylate cyclase. When it was shown that cGMP did not mediate the actions of insulin and that guanylate cyclase existed in both membrane-bound and -soluble forms and that its regulation was nothing like that of adenylate cyclase, cGMP became, in essence, an orphan messenger. For many years cGMP remained an orphan signal because no conventional ligands could be found that could activate guanylate cyclase. cGMP was shown eventually to mediate visual signals in rod cells in the retina (described above), but this was considered to be an exception since light was not a conventional ligand and the cGMP did not activate a protein kinase; rather, it held open a sodium channel in the rod cell membrane. One reason for the difficulty in understanding guanylate cyclase signalling was that activating ligands in mammals had not yet been discovered. It was not until atrial natriuretic factor was identified in 1981 [73] that it was possible to identify a conventional ligand for the membrane-bound form of guanylate cyclase in mammalian cells. The discovery that NO and CO are physiological messengers made it possible to describe ligands, albeit unconventional ones, for the soluble form of guanylate cyclase. Because our mindset was so focused on the way that adenylate cyclase and cAMP were regulated, our expectations for the regulation of guanylate cyclase/cGMP were misdirected, and the understanding of this signalling system was, therefore, greatly delayed.

A similar lesson is that sometimes we do not find what we are looking for because we do not use the right methods. This is illustrated by the example of the protein phosphatases described earlier; protein phosphatases were not considered to be regulated intracellular messengers because they were first studied in such a way that the enzymes autoactivated during tissue processing and thus appeared to be constitutively active. Further, protein kinases were considered to be carefully controlled by the cells so it was not expected that protein phosphatases would need to be regulated. To say this another way, if we only look for signalling by protein kinases, we will certainly find it, but we will miss all the other mechanisms of signalling such as signalling by protein phosphatases or direct activation of channels by cyclic nucleotides.

One final lesson to consider about both science, in general, and the field of signal transduction, in particular, is that we have to keep an open mind. The most seemingly outlandish combinations of signalling factors of which anyone could dream have already been conceived and utilized by nature. For example, before the identification of the regulation of PKC, who would have believed that membrane lipids could serve as signalling mediators? Now we know that phospholipases A and D as well as the sphingomyelin pathway also activate lipid/lipid-derived second messengers. As the story of the signalling cascades activated by the phosphatidylinositol 3-kinases unfolds (see Fig. 6; [38]), the role of lipid mediators becomes even more intriguing. Who would have dreamed that poisonous gases such as NO or CO could have a physiological role as signalling molecules? Yet this has clearly been established.

What does the future hold? Undoubtedly, new signal transduction pathways will be described, and the black boxes in the known pathways will become illuminated. The concept of tyrosine phosphorylation was an exciting new idea when first described in the late 1970s [74–76]. The role of tyrosine phosphorylation in cellular signalling has become firmly entrenched along with that of serine and threonine phosphorylation; now we have the compelling prospect of histidine phosphorylation as a signalling event in eukaryotic cells [77]. It is likely that ligands that are not traditional ligands will continue to receive increasing interest in their ability to activate signal transduction responses. For example, shear stress in blood vessel walls [78] and oxidative stress [31] are not traditional ligands, but these physical events trigger signalling pathways in the same way as more conventional ligands. Hopefully the concepts presented in this primer will prove useful in deciphering the known and emerging signalling pathways. Eventually, the tangled web of signal transduction pathways will become straight.

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