Role of Inositol Lipid Breakdown in the Generation of Intracellular Signals
State of the Art Lecture
JOHN R. WILLIAMSON

SUMMARY Many hormones, neurotransmitters, and secretagogues act by increasing the intracellular free Ca\(^{2+}\) concentration in target cells. The initial event following binding of agonists to specific receptors in the plasma membrane involves a receptor-mediated activation of a guanosine nucleotide-binding protein (G protein), which induces a Ca\(^{2+}\)-independent activation of phospholipase C. This novel, presently uncharacterized G protein is inactivated by pertussis toxin-catalyzed adenosine S'-diphosphate ribosylation in some but not all cell types. Phospholipase C catalyzes the breakdown of inositol lipids, notably phosphatidylinositol 4,5-bisphosphate, with the production of inositol phosphates and 1,2-diacylglycerol. Inositol 1,4,5-trisphosphate (IP\(_3\)) is responsible for a rapid mobilization of intracellular Ca\(^{2+}\) by activating Ca\(^{2+}\) efflux from a subpopulation of the endoplasmic reticulum.

The properties of this process are consistent with its being a ligand-activated ion channel with electrogenic Ca\(^{2+}\) efflux being charge-compensated by K\(^+\) influx. Sustained hormonal responses require extracellular Ca\(^{2+}\) and a prolonged elevation of the cytosolic free Ca\(^{2+}\). This is brought about by hormone-mediated changes of Ca\(^{2+}\) flux across the plasma membrane involving both an inhibition of Ca\(^{2+}\) efflux and an activation of Ca\(^{2+}\) influx. This review summarizes recent findings concerning the role of G proteins in receptor coupling to phospholipase C; the regulation of enzymes of phosphoinositol metabolism; the evidence for IP\(_3\) being a Ca\(^{2+}\)-mobilizing second messenger and its mechanism of action; the formation of new inositol phosphates and their possible significance; the relation of intracellular Ca\(^{2+}\) mobilization and plasma membrane Ca\(^{2+}\) fluxes to the kinetics of the hormone-induced cytosolic free Ca\(^{2+}\) transient; and the possible roles of protein kinase C in influencing the hormone-mediated functional response. (Hypertension 8 [Suppl II]: 11-140—11-156, 1986)

KEY WORDS • inositol trisphosphate • inositol tetrakisphosphate • calcium mobilization • guanosine S'-triphosphate-binding proteins • protein kinase C • inositol lipid metabolism • calcium-mobilizing hormones • phosphatidylinositol

The regulation of different characteristic and specialized cell functions is brought about by the binding of agonists or hormones to specific receptor proteins located in the plasma membrane. The complex process of information transfer from chemicals in the extracellular environment to the regulation of intracellular enzymes and proteins is achieved by a number of different signaling mechanisms. The best-understood example of such a process is the β-adrenergic activation of adenylyl cyclase, which causes an increased production of cyclic adenosine 3',5'-monophosphate (cAMP) as a second messenger. The subsequent activation of cAMP-dependent protein kinase results in the phosphorylation and modulation of the activity of a variety of target enzymes and regulatory proteins within the cell, with consequent alterations of specific cell functions.

Cell function can also be modulated by receptor-activated mechanisms that do not involve cAMP but instead are mediated by increases of the free Ca\(^{2+}\) concentration in the cytosol. Calcium causes changes in the activity of a variety of proteins, including protein kinases and phosphatases, either directly or after binding to calmodulin or other Ca\(^{2+}\)-binding proteins. Until recently, however, the source of the Ca\(^{2+}\), the amount and kinetics of the Ca\(^{2+}\) changes, and the mechanism of hormone-stimulated cellular Ca\(^{2+}\) mobilization were largely unknown.
Many studies have now established that a wide range of compounds, including hormones, neurotransmitters, secretagogues, chemoattractants, and other cell-activating substances that involve Ca\(^{2+}\) mobilization in the expression of the biological response, cause an activation of a phosphodiesterase, termed phospholipase C, which breaks down inositol lipids in the plasma membrane.\(^9,10\) However, unlike receptor-mediated activation of adenylate cyclase, which produces cAMP as the only second messenger, receptor-activated inositol lipid breakdown serves a dual signaling role with production of two second messengers having different functions.\(^10-13\) One of these compounds — namely, inositol-1,4,5-trisphosphate (IP\(_3\)) — is responsible for eliciting intracellular Ca\(^{2+}\) mobilization, and the second compound, 1,2-diacylglycerol, has as its primary signaling role the activation of a phospholipid-dependent protein kinase in the plasma membrane called protein kinase C.\(^14\) Hence, in principle, agents that interact with inositol lipid metabolism not only cause Ca\(^{2+}\) release with subsequent phosphorylation of proteins by Ca\(^{2+}\)-dependent protein kinases or dephosphorylation by Ca\(^{2+}\)-dependent phosphoprotein phosphatases, but also cause increased phosphorylation of a different set of proteins by activation of protein kinase C.

Another role of diacylglycerol, prevalent in platelets, is to serve as a precursor for arachidonic acid release through sequential degradation by diglyceride and monoglyceride lipases. In other cells (e.g., neutrophils) the production of arachidonic acid may be secondary to Ca\(^{2+}\) release through activation of phospholipase A\(_2\). Arachidonic acid is the predominant fatty acid in the second position of the glycerol moiety of the inositol lipids and is metabolized to a variety of eicosanoid mediators (prostaglandins, thromboxanes, and leukotrienes). These metabolites can function as intercellular mediators to initiate responses in cells other than those in which they are produced, as in the production of prostaglandin I\(_2\) and leukotrienes by endothelial cells, which cause vasodilation and vasoconstriction, respectively, in smooth muscle, as well as effects on platelet aggregation.\(^15,16\) Prostaglandin H\(_2\) and thromboxane A\(_2\) interact with cell surface receptors in platelets that are themselves coupled to phospholipase C and hence may produce a cascade-signaling effect by reinforcing the Ca\(^{2+}\)-mobilizing response of the original agonist.\(^17-19\)

The objective of this article is to summarize recent developments and conclusions regarding the regulation of receptor-activated inositol lipid metabolism, the role of IP\(_3\) in Ca\(^{2+}\) mobilization, the kinetics of agonist-induced changes of cytosolic free Ca\(^{2+}\), and the roles of Ca\(^{2+}\) and protein kinase C in the mediation of the overall tissue response.

**Inositol Lipid Metabolism and Ca\(^{2+}\) Mobilization**

The inositol lipids are rather minor components of the plasma membrane, representing 5 to 10% of the total phospholipids, but are the most metabolically active. The major one is phosphatidylinositol (PI).

Each of two phosphorylated derivatives, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-biphosphate (PIP\(_2\)), contains about 1% of the total inositol lipids. The currently accepted view of the role of inositol lipid metabolism in Ca\(^{2+}\) mobilization is summarized in Figure 1. IP\(_3\) is formed by the cleavage of the phosphodiesterase linkage between the third position of the glycerol moiety of PIP, and the first position of the inositol ring after phospholipase C has been activated by the binding of the agonist to its receptor. Activation of phospholipase C is probably mediated by a guanosine 5'-triphosphate (GTP)-binding coupling protein (labeled G\(_x\), in Figure 1), although its identity has not yet been ascertained.\(^20\) IP\(_3\) is released into the cytosol and activates the efflux of Ca\(^{2+}\) from an intracellular, nonmitochondrial, vesicular calcium pool. This Ca\(^{2+}\)-signaling system is terminated by hydrolysis of IP\(_3\) to inositol-1,4,6-trisphosphate (IP\(_{6}\)), which is inactive in causing Ca\(^{2+}\) release.

**Role of GTP-Binding Proteins in Receptor Coupling**

A rapidly growing number of hormone receptors have been isolated and purified, and their structural and functional properties investigated in detail. These receptor proteins, which may be in the form of monomeric or oligomeric subunits, span the phospholipid bilayer of the plasma membrane and contain functionally active sites on the inner side of the membrane, as well as ligand-binding sites on the outer membrane surface. Information transfer through the plasma membrane is probably mediated by a conformational change, which alters the properties of the inward-facing regions of the receptor protein. Hormone activa-
tion of some receptors, such as those for insulin and various growth factors, induces tyrosine kinase activity, which is thought to cause a secondary activation of serine- and threonine-specific protein kinases for the mediation of at least some of their functional effects. Other receptors interact with specialized GTP-binding proteins in the plasma membrane as part of the receptor-effector coupling mechanism. In fact, some receptors may elicit multiple signaling mechanisms, since it has been suggested that the insulin receptor also interacts with a specific GTP-binding protein, which mediates certain effects of insulin on target cells.

A question of fundamental importance in understanding the mechanism of hormone activation in the inositol lipid signaling system is whether a GTP-binding protein is involved in the activation of phospholipase C. Before this question is addressed, it is useful to summarize current knowledge concerning the role of GTP-binding proteins in the receptor-mediated activation of adenylate cyclase.

Receptor-mediated stimulation (e.g., by β-adrenergic agonists) or inhibition (e.g., by α-adrenergic agonists or muscarinic agonists acting on M2 receptors) of adenylate cyclase activity is mediated by two different GTP-binding proteins, Gs (stimulatory) and Gi (inhibitory), which belong to a family of structurally and functionally related proteins. In nonstimulated cells, these proteins are thought to be present in the membrane as inactive αβγ-subunit oligomeric complexes with guanosine 5’-diphosphate (GDP) bound to the α subunit. In the case of Gi and Gs, the α-subunits are different (relative molecular weight, 52,000 or 45,000 for αs and 41,000 for αi), whereas the β-subunit (relative molecular weight, 35,000) and the γ subunit (relative molecular weight, 5000–10,000) are very similar. Receptor activation causes dissociation of the α-subunit, accompanied by a replacement of bound GDP by GTP. Activation of adenylate cyclase occurs when αs-GTP binds to the enzyme. This stimulatory signal is terminated when intrinsic GTPase activity of the subunit, followed by reassociation of αs-GDP with the β- and γ-subunits. Inhibition of activated adenylate cyclase by Gi, on the other hand, is thought to occur either by a direct inhibitory effect of the αi-subunit or by a decrease in the amount of the free αs-subunit as a consequence of an increased availability of β-subunits upon dissociation of Gi.

In addition to Gi and Gs, two other GTP-binding proteins have been isolated and purified. The function of one, called transducin (with an α-subunit relative molecular weight of 39,000), which so far has been found only in photoreceptors, is to allow vertebrate rhodopsin to stimulate a cyclic guanosine 3’,5’-monophosphate (cGMP) phosphodiesterase upon photoactivation. A fourth GTP-binding protein, termed Gq, has been purified from brain. It has an α-subunit with a relative molecular weight of 39,000, but its βγ-subunits are apparently identical to those of Gi and Gs. The function of Gq has not yet been elucidated, but recent voltage-clamp studies with atrial cells suggest that it may have a special signaling role in activating an inwardly rectifying K+ channel through muscarinic M2 receptors.

It is highly likely that cells contain other, as yet uncharacterized GTP-binding proteins of a similar nature, which activate or inhibit specific enzymes through a receptor-effector coupling mechanism.

A number of approaches and tools have been used to identify and study the functional roles of these GTP-binding proteins. One approach is the use of specific bacterial toxins that catalyze adenosine 5’-diphosphate (ADP) ribosylation at particular sites on the α-subunits of the GTP-binding proteins by transfer of the ADP-ribose moiety from nicotinamide adenine dinucleotide (oxidized form). Cholera toxin causes ADP ribosylation of Gs, with stimulation of the release of GDP from the αs-subunit and inhibition of agonist-stimulated GTPase activity, so that there is persistent activation of adenylate cyclase. Bordetella pertussis toxin causes ADP ribosylation of Gi and uncouples inhibitory receptors from the protein, so that the normal agonist stimulation of GTPase activity is prevented, with the result that the inhibitory effects of Gi on adenylate cyclase are abolished. A second approach that has been used to investigate the role of GTP-binding proteins in signal transfer is the addition of nonhydrolyzable analogues of GTP to permeabilized cells, plasma membrane preparations, or reconstituted systems. These GTP analogues stabilize the α-subunits in the active form, since GTPase activity is prevented. A third approach focuses on the interaction of agonists, receptors, and GTP-binding proteins. A shift of agonist binding from a high-affinity to a low-affinity state can be induced by activation of the GTP-binding coupling protein with GTP or its analogues. Low-affinity binding sites correspond to a binary complex of agonist and receptor, whereas high-affinity sites are associated with a ternary complex of agonist, receptor, and GTP-binding protein.

The first indications that a GTP-binding protein may function as a signal transduction mechanism for Ca2+-mobilizing agonists were reports that GTP or its nonhydrolyzable analogues decreased the affinity of various receptors for binding to their agonists. Some examples of this effect are norepinephrine binding to α1-receptors, carbachol interactions with muscarinic receptors, chemotactic peptide binding to neutrophil membranes, and vasopressin and angiotensin II binding to liver plasma membranes. More conclusive evidence indicating that a GTP-binding protein may be specifically involved in inositol lipid metabolism has been obtained recently from a number of laboratories. After nonhydrolyzable GTP analogues had been introduced into mast cells, the addition of extracellular Ca2+ caused an increased secretion of histamine without any change of cAMP levels. Similarly, addition of GTP or its analogues to permeabilized platelets decreased the Ca2+ requirements for serotonin release and promoted the formation of diacylglycerol, indicating a stimulation of phospholipase C. In more recent work with homogenates and...
The identity of the GTP-binding protein that apparently interacts with phospholipase C has not been ascertained. In a number of cells, including polymorphonuclear leukocytes and neutrophils, human leukemic HL60 cells, and the hybrid cell line WBC-264-9C, chemotactic peptide–induced interactions with inositol lipid metabolism, arachidonic acid release, O₂⁻ generation, Ca²⁺ mobilization, and other responses were greatly inhibited by pretreatment of the cells with pertussis toxin. In these cells it was shown that the effects induced by the chemotactic peptide fMet-Leu-Phe were mediated not by changes in cAMP but rather by a receptor-coupled activation of phospholipase C. However, at concentrations higher than those required for maximum cellular effects on human neutrophils, fMet-Leu-Phe was shown to inhibit the forskolin-induced increase of adenylyl cyclase in neutrophil plasma membranes, with the effect being abolished by pertussis toxin pretreatment. The attenuation of all these chemotactic peptide–induced effects by pertussis toxin was associated with ADP ribosylation of a single membrane-bound protein (relative molecular weight, 41,000) characterized as Gᵢ. The involvement of Gᵢ in coupling with the chemotactic peptide receptor in neutrophils was substantiated by further studies showing that the addition of Gᵢ (isolated from brain) to pertussis toxin–pretreated membranes was able to restore fMet-Leu-Phe–induced GTase activity, which had been inhibited by the pertussis toxin treatment. The conclusion reached from these studies was that chemotactic peptide receptors in neutrophils and similar cells are coupled to Gᵢ, and that this G protein has a bifunctional role in inhibiting adenylyl cyclase and activating inositol lipid metabolism. In other cell types, however, pertussis toxin treatment apparently has no effect in inhibiting the actions of Ca²⁺–mobilizing hormones. Thus, pertussis toxin pretreatment had no observed effect on the binding of α₁-adrenergic ligands to rat kidney cortex membranes or of muscarinic ligands to receptors of astrocytoma cells in the absence or presence of guanine nucleotides. More directly, it has been shown that the addition of GTP analogues alone or together with an agonist causes an increased breakdown of polyphosphoinositides and formation of inositol phosphates. The role of the stabilized (GTP-bound) α-subunit of GTP-binding protein may be to decrease the Ca²⁺ requirement for activation of phospholipase C.

The involvement of Gᵢ in coupling with phospholipase C has been shown to prevent α₁-adrenergic stimulation of PI turnover in subsequently isolated adipocytes. In contrast, pertussis toxin had no observed effect on α₁-adrenergic stimulation of respiration in brown adipocytes. In these studies, pertussis toxin augmented β-adrenergic effects on lipolysis and respiration and abolished the inhibitory effects of adenosine. There was an extensive ADP ribosylation of a membrane protein with a relative molecular weight of 41,000, indicating that the inhibitory influence of Gᵢ on adenylyl cyclase was prevented.

On the basis of currently available data, it is clear that receptor coupling to phospholipase C is mediated by a GTP-binding protein. The evidence is strong in neutrophils and similar cells that the G protein is ADP-ribosylated and inactivated by pertussis toxin and that the ribosylated subunit has a molecular weight very similar to that of the α-subunit of Gᵢ. In other cells, however, the G protein involved in Ca²⁺ mobilization appears not to be affected by pertussis toxin, suggesting that it is distinct from Gᵢ. Thus, at present it is not clear whether there are fundamental differences between different cell types in the nature of the G protein that couples receptors to phospholipase C or whether there is a similar, as yet unidentified, G binding protein that differs from Gᵢ in its susceptibility to ribosylation and inactivation by pertussis toxin in different cells but that uniquely couples to phospholipase C in all cells. In leukocytes the concanavalin A–induced increase of cytosolic free Ca²⁺, unlike that of chemoattractant peptides, is not sensitive to inhibition by pertussis toxin. It is possible, therefore, that specific receptors in certain cells form a complex with the phospholipase C–coupled G protein to make it unsusceptible to ribosylation and inactivation by pertussis toxin.

Enzymes of Phosphoinositide Metabolism

The inositol lipids in the cell are in a continuous state of turnover, as revealed by labeling studies with [³H]inositol and [³²P]phosphate. The recognized pathways of phosphoinositide metabolism are depicted in Figure 2. Two different types of enzymes are involved in phosphoinositide hydrolysis — phosphomonoesterases and phospholipase C. The phosphomonoesterase-
ases selectively remove phosphate from the fourth and fifth positions of the inositol ring and convert PIP$_2$ to PIP$_3$, and the latter to PI. Together with PI and PIP kinases, these enzymes are responsible for the turnover of the phosphate in the fourth and fifth positions of the inositol ring, without affecting the mass of these inositol lipids in the steady state. Phospholipase C causes hydrolysis of all three inositol lipids, with the production of the common product 1,2-diacylglycerol and the liberation of IP$_3$, IP$_2$, and IP$_1$, from PI, PIP, and PIP$_2$, respectively (see Figure 2). Various phosphatases are responsible for the successive hydrolysis of IP$_3$ to IP$_2$, IP$_2$ to IP$_1$, and IP$_1$ to inositol. The IP$_3$ and IP$_2$ phosphatases, but not the IP$_1$ phosphatase are sensitive to inhibition by Li$^+$, and all the phosphatases require Mg$^{2+}$ for activity. Li$^+$ is often added to cell incubations to promote accumulation of the inositol phosphates and aid their measurement. In most cells diacylglycerol is metabolized primarily to phosphatidic acid by diacylglycerol kinase and subsequently to cytidine 5'-diphosphate-diacylglycerol, which condenses with inositol to reform PI. The incorporation of $^{32}$P from $[^32]$P-γ-adenosine 5'-triphosphate (ATP) into PA of whether hormone-stimulated hydrolysis of the polyphosphoinositides was secondary to an elevation of cytosolic free Ca$^{2+}$ resulting from increased entry of Ca$^{2+}$ into the cell. In the case of iris smooth muscle, it was later demonstrated that the $\alpha_1$-adrenergic antagonist prazosin prevented increases of IP$_3$ induced by A23187, suggesting that the breakdown of PI$_3$ was secondary to release of norepinephrine by the ionophore. In other cells (e.g., mast cells and platelets) A23187 has been shown to stimulate arachidonic acid release, probably by direct Ca$^{2+}$ activation of phospholipase A$_2$, with secondary activation of phospholipase C by cyclo-oxygenase products of arachidonate metabolism. Thus, although there are quantitative and perhaps some qualitative differences in the response of different tissues, most of the available data are consistent with the conclusion that the initial effect of agonist stimulation is to increase the breakdown of the polyphosphoinositides and that IP$_3$-induced Ca$^{2+}$ mobilization may subsequently cause an increased rate of hydrolysis of the much more abundant PI. The main function of phospholipase C-activated PI hydrolysis, therefore, would be to generate diacylglycerol.

The relative amounts of diacylglycerol and IP$_3$ formed during hormonal stimulation of cells are likely to be important for expression of the separate signaling roles of these compounds because of the different sensitivities of their effects. Thus, a relatively mild agonist-induced stimulation of phospholipase C activity may produce enough IP$_3$ to elicit mobilization of Ca$^{2+}$ but not enough diacylglycerol to activate protein kinase C fully. Expression of the diacylglycerol signaling role, therefore, may require simultaneous hydrolysis of PI and particularly PI$_3$. Conversely, it is possible that some tissues, such as cardiac muscle, may lack the Ca$^{2+}$ mobilization branch of the signaling system because of the absence or low sensitivity of the IP$_3$ receptor in the sarcoplasmic reticulum while retaining the diacylglycerol-activated protein kinase C branch. Vascular smooth muscle and frog skel-
nal muscle have been shown to exhibit IP$_3$-induced Ca$^{2+}$ release from intracellular storage sites. Whether IP$_3$ is involved in excitation-contraction coupling by a direct activation of phospholipase C is questionable, however, since IP$_3$ was found to be ineffective in releasing Ca$^{2+}$ from cardiac and skeletal muscle sarcoplasmic reticulum vesicles.

A continuous agonist-mediated stimulation of IP$_3$ breakdown would rapidly lead to its depletion and, consequently, to a cessation of IP$_3$ production and intracellular Ca$^{2+}$ signaling without replenishment by PIP$_2$ kinase. It is known that long-term hormonal stimulation of cells such as hepatocytes results in a sustained depletion of the IP$_3$-sensitive Ca$^{2+}$ pool and that IP$_3$ levels remain elevated as long as the hormone is present. After the initial fall of IP$_3$, however, it gradually returns to control levels, suggesting a stimulation of PIP$_2$ kinase. Kinetic studies using $[^{32}P]$phosphate labeling with platelets have in fact demonstrated an increase of flux through PIP$_2$ kinase in conjunction with a thrombin-stimulated breakdown of IP$_3$. Relief of the inositol lipid kinases from product inhibition, as demonstrated for PIP$_2$ kinase, may also account for an increase of flux through the kinases relative to hydrolysis by the phosphomonoesterases. In the kinetic study with platelets, however, it was shown that flux from PI to PIP$_2$ was too small to account for the observed rate of breakdown of PI during thrombin stimulation, suggesting a larger breakdown of PI through phospholipase C than conversion to PIP$_2$ by PI kinase. Further studies with different cells are required before the regulation of PIP$_2$ and PIP$_3$ levels in the plasma membrane and flux through the different enzymes can be defined in more detail.

Criteria for a Direct Role of Inositol Lipid Metabolism in Ca$^{2+}$ Mobilization

To establish that a hormone-stimulated breakdown of polyphosphoinositides and formation of IP$_3$, a number of criteria have to be fulfilled. The most important are the following: 1) Hydrolysis of PIP$_2$ and formation of IP$_3$ should not be secondary to the rise of cytosolic free Ca$^{2+}$. 2) PIP$_3$ breakdown and IP$_3$ formation should show a sensitivity to the hormone concentration similar to that for the increase of cytosolic free Ca$^{2+}$. 3) Removal of the agonist should cause a prompt fall of IP$_3$ levels and cytosolic free Ca$^{2+}$. 4) IP$_3$ at concentrations equivalent to those reached in the intact cell by hormone stimulation should cause Ca$^{2+}$ release when injected into cells or added to cell-free preparations or permeabilized cells.

In most tissues where IP$_3$ is thought to be important in releasing Ca$^{2+}$, these criteria have not all been rigorously tested, but there is little doubt that stimulus-induced breakdown of PIP$_3$, formation of IP$_3$, and release of Ca$^{2+}$ from nonmitochondrial intracellular Ca$^{2+}$ stores represent a physiological signaling mechanism in a wide variety of cells.

Evidence That IP$_3$ Is a Ca$^{2+}$-Mobilizing Second Messenger

The evidence supporting the second-messenger role of IP$_3$ in liver and other tissues has been reviewed in detail elsewhere. Various elegant experiments have now shown that injection of IP$_3$ into single cells causes a release of Ca$^{2+}$ from an intracellular pool. Thus, an increase of cytosolic free Ca$^{2+}$ has been obtained by injection of IP$_3$, into Limulus photoreceptors, together with the elicitation of electrical events similar to those produced by light. Similarly, injection of IP$_3$ has been shown to cause the release of Ca$^{2+}$ from an IP$_3$-sensitive Ca$^{2+}$ pool, which elicits the Ca$^{2+}$-mediated activation of sea urchin eggs. Studies with hepatocytes have shown that the rate of breakdown of PIP$_2$ and the rate of formation of IP$_3$ are compatible with the hypothesis that IP$_3$ is directly responsible for the observed increase of cytosolic free Ca$^{2+}$. The formation of IP$_3$, in concert with a rise of cytosolic free Ca$^{2+}$ by suitable agonists has now been demonstrated in many different cell types. In a recent study with permeabilized pancreatic acinar cells that retain their ability to respond to muscarinic agonists by activating inositol lipid metabolism, it was shown that hormone-induced IP$_3$ production was directly proportional to the amount of Ca$^{2+}$ released from the intracellular pools under a variety of conditions.

Convincing evidence that IP$_3$ is the causative agent in intracellular Ca$^{2+}$ mobilization was first obtained by addition of IP$_3$ to saponin-permeabilized cells incubated in a high-K$^+$ medium supplemented with ATP and low concentrations of Ca$^{2+}$. The typical response is a rapid release of Ca$^{2+}$, followed by a slower resequestration of the liberated Ca$^{2+}$ into the intracellular organelles until the medium-free Ca$^{2+}$ concentration falls to a steady state value of about 150 nM (Figure 3A). This response is very sensitive to IP$_3$, which shows half-maximum and maximum effects at 0.1 nM and 0.5 nM, respectively, with hepatocytes, although other cell types appear to be somewhat less sensitive. Clearly, for this effect to have physiological consequences in any particular tissue, it must be demonstrated that the hormone-induced increase of IP$_3$ is sufficient to cause the required amount of Ca$^{2+}$ mobilization to elicit the biological response. This has now been shown for a number of tissues, including hepatocytes and platelets.

The nature of the intracellular organelle responsible for IP$_3$-induced Ca$^{2+}$ release was ascertained by the addition of IP$_3$ to permeabilized cells in which the calcium content of the endoplasmic reticulum or the mitochondria was selectively controlled by the presence of suitable inhibitors of Ca$^{2+}$ uptake. These experiments showed that Ca$^{2+}$ was mobilized exclusively from a nonmitochondrial pool. More directly, isolated mitochondria from which all reticular material had been removed have been shown to be inactive in releasing Ca$^{2+}$ upon addition of IP$_3$, whereas mitochondria-free preparations of endoplasmic reticulum retain the ability to release Ca$^{2+}$ with IP$_3$, Like-
Mechanism of IP$_3$-Induced Ca$^{2+}$ Release

Relatively little is known about the mechanism of IP$_3$-induced Ca$^{2+}$ release. It is presumably mediated by the binding of IP$_3$ to a specific protein in the endoplasmic reticulum membrane, but this putative IP$_3$ receptor has not yet been identified or characterized. However, an important advance in this direction has been made by Hirata et al., who synthesized an arylazide photoaffinity derivative of IP$_3$ and showed that it caused an irreversible inhibition of IP$_3$-induced Ca$^{2+}$ release in permeabilized photolabeled macrophages. Calcium release from permeabilized cells or preparations of endoplasmic reticulum is highly specific for the natural product of the PIP$_3$ breakdown, and the presence of phosphates in the fourth and fifth positions of the inositol ring appears to be essential for activity. The phosphate in the first position may enhance the binding of IP$_3$ to the IP$_3$ receptor. It is presumably mediated by a GTP-binding protein or by phosphorylation would cause an increase of IP$_3$ levels in the absence of any direct stimulation of phospholipase C activity.

The location of the subcellular structures containing the IP$_3$-sensitive calcium pool within the cell has not been ascertained. In liver and several other, but not all, cell types the maximal amount of Ca$^{2+}$ released by IP$_3$ is considerably less than the total calcium content of the endoplasmic reticulum calcium pool. It remains possible that the IP$_3$-sensitive Ca$^{2+}$ pool is located in a specialized subpopulation of the endoplasmic reticulum, with a defined spatial location in the cell (e.g., adjacent to the plasma membrane). Alternatively, the intracellular vesicular structures containing the IP$_3$-releasable Ca$^{2+}$ may be distributed heterogeneously within the cell rather than in a single homogeneous calcium pool. Recent studies have used iontophoresis to introduce IP$_3$ into frog (Xenopus laevis) eggs and have recorded the elicited Ca$^{2+}$ response by Ca$^{2+}$-selective microelectrodes. These studies show that although both shallow and deep injections of low concentrations of IP$_3$ trigger local increases of intracellular free Ca$^{2+}$, only shallow injections elicit a Ca$^{2+}$-triggered Ca$^{2+}$ release, which accompanies activation of physiological responses. This major release of Ca$^{2+}$, which is much greater than the IP$_3$-induced Ca$^{2+}$ release, is thought to be derived from a calcium pool adjacent to the egg cortex. The fact that IP$_3$ injections cause small pulses of Ca$^{2+}$ release independently of the site of injection suggests that the IP$_3$-sensitive calcium pool is distributed throughout the cell and that diffusion of IP$_3$ or Ca$^{2+}$ limits the physiological response.

Mechanism of IP$_3$-Induced Ca$^{2+}$ Release

Relative little is known about the mechanism of IP$_3$-induced Ca$^{2+}$ release. It is presumably mediated by the binding of IP$_3$ to a specific protein in the endoplasmic reticulum membrane, but this putative IP$_3$ receptor has not yet been identified or characterized. However, an important advance in this direction has been made by Hirata et al., who synthesized an arylazide photoaffinity derivative of IP$_3$ and showed that it caused an irreversible inhibition of IP$_3$-induced Ca$^{2+}$ release in permeabilized photolabeled macrophages. Calcium release from permeabilized cells or preparations of endoplasmic reticulum is highly specific for the natural product of the PIP$_3$ breakdown, and the presence of phosphates in the fourth and fifth positions of the inositol ring appears to be essential for activity. The phosphate in the first position may enhance the binding of IP$_3$, since glycerophosphatidylinositol-4,5-P$_2$ and inositol-2,4,5-P$_3$ are about 10-fold less effective than IP$_3$ in releasing Ca$^{2+}$. However, more knowledge concerning the structural requirements for IP$_3$ analogues and potential inhibitors is urgently needed. Calcium release induced by IP$_3$ is not impaired by vandate inhibition of Ca$^{2+}$-ATPase and does not require ATP, indicating that Ca$^{2+}$ release does not occur by reversal of Ca$^{2+}$-ATPase uptake. Continuous exposure to IP$_3$ does not induce desensitization of the
Ca\(^{2+}\) release mechanism, which is also not affected by external Ca\(^{2+}\) concentrations up to 100 \(\mu\)M.\(^{103}\) The rate of IP\(_3\)-induced Ca\(^{2+}\) release at 37\(^\circ\)C is on the order of 50 nmol/sec/mg of “active” endoplasmic reticulum, and the effect of lowering the temperature to 4\(^\circ\)C is negligible.\(^{128}\) The currently known properties of IP\(_3\)-mediated Ca\(^{2+}\) release are consistent with the proposal\(^{125}\) that the binding of IP\(_3\) to its receptor regulates a Ca\(^{2+}\) channel in specialized regions of the endoplasmic reticulum membrane and that charge compensation is achieved by an inward movement of K\(^+\).\(^{1,2}\) (S.K. Joseph and J.R. Williamson, submitted for publication, 1986).

**Kinetics of IP\(_3\) Formation and Different Isomers**

Most measurements of the accumulation of inositol phosphates in cells after agonist stimulation have been made using cells prelabeled with \(^{3}H\)inositol, and the different \(^{3}H\)-labeled intermediates have been separated by Dowex resin ion-exchange chromatography. With this technique, it has become apparent that different Ca\(^{2+}\)-mobilizing agonists acting on the same cell stimulate phospholipase C to different degrees and that a similar maximum Ca\(^{2+}\) mobilization can be achieved with different accumulations of IP\(_3\).\(^{133,134}\) Thus, although at saturating concentrations vasopressin and the \(\alpha_1\)-adrenergic agent phenylephrine cause a similar rapid increase of cytosolic free Ca\(^{2+}\), phenylephrine causes only a 30% increase of IP\(_3\) levels, whereas vasopressin results in almost a twofold increase, which does not peak until after about 5 minutes. These observations were initially interpreted on the basis of sufficient IP\(_3\) being produced in the first few seconds of agonist addition to cause maximum Ca\(^{2+}\) release from the IP\(_3\)-sensitive Ca\(^{2+}\) pool, with the later accumulation of IP\(_3\) representing overproduction.\(^{113}\)

Recent studies with parotid gland\(^{129,130}\) and hepatocytes\(^{131,134}\) have shown that two isomers of IP\(_3\) are formed during hormonal stimulation — namely, inositol-1,3,4-P\(_3\), and inositol-1,4,5-P\(_3\). Measurements of the kinetics of changes in the two isomers after stimulation of cells with Ca\(^{2+}\)-mobilizing agonists showed that inositol-1,3,4-P\(_3\) was produced after the onset of inositol-1,4,5-P\(_3\) formation and continued to accumulate for several minutes, whereas inositol-1,4,5-P\(_3\) fell to a new steady state level after peaking at 20 seconds. At present, no functional effects have been attributed to the inositol-1,3,4-P\(_3\) isomer. The two observations — that it does not start to accumulate until after the peak of the intracellular Ca\(^{2+}\) transient and that the small elevation in the steady state level of the cytosolic free Ca\(^{2+}\) falls abruptly upon addition of hormone antagonist, whereas total IP\(_3\) levels fall relatively slowly — suggest that the inositol-1,3,4-P\(_3\) isomer is not active in Ca\(^{2+}\) mobilization. In pancreatic acinar cells\(^{132}\) and hepatocytes,\(^{133}\) high concentrations of Li\(^+\) (20 mM) were found to cause an increase in the agonist-stimulated accumulation of total IP\(_3\), which was paradoxical, since the inositol-1,4,5-P\(_3\) phosphatase was not affected by Li\(^+\).\(^{131,132}\) Measurements of the IP\(_3\) isomers in pancreatic acinar cells and hepatocytes have subsequently shown that with Li\(^+\) pretreatment, only the inositol-1,3,4-P\(_3\) isomer shows a further accumulation.\(^{131,134}\) indicating that only the IP\(_3\) phosphatase specific for this isomer is Li\(^+\)-sensitive. Very recently, higher phosphorylated forms of inositol have been detected in stimulated cells.\(^{113,133,134}\) In carbachol-stimulated brain slices inositol-1,3,4,5-tetrakisphosphate (IP\(_4\)) is produced as rapidly as inositol-1,4,5-P\(_3\), and before inositol-1,3,4,5-P\(_3\) has reached a peak (5 seconds).\(^{134}\) Of the two possibilities — that IP\(_3\) is formed by hydrolysis of phosphatidylinositol-3,4,5-P\(_3\), or by a soluble inositol-1,4,5-P\(_3\) kinase — the latter is more likely, since animal cells (as well as avian erythrocytes\(^{135}\)) contain inositol-1,4,5-P\(_3\) kinase activity.\(^{134}\) In any case, IP\(_3\) seems to be the precursor for the inositol-1,3,4,-P\(_3\) isomer,\(^{133}\) and presumably has an important second-messenger role in stimulus-response coupling.

Early studies showed that the product of PI metabolism by phospholipase C consisted of a mixture of inositol-1-phosphate (IP\(_1\)) and inositol-1,2-(cyclic) phosphate, with the latter compound being converted to IP\(_3\) by a tissue hydrolase before further hydrolysis to inositol.\(^{136}\) Interest in the cyclic inositol phosphates waned after it was shown that inositol-1,2-(cyclic) phosphate was unable to elicit Ca\(^{2+}\) mobilization.\(^{137}\) However, it has recently been shown\(^{138}\) that hydrolysis of PIP and PIP\(_2\), by purified phospholipase C is associated with the formation of inositol-1,2-(cyclic)-4-phosphate and inositol-1,2-(cyclic)-4,5-bisphosphate products. The cyclic and noncyclic forms of inositol-1,4,5-P\(_3\), released Ca\(^{2+}\) from saponin-permeabilized platelets with approximately equal sensitivity, but the cyclic form was apparently more effective in inducing a change in membrane conductance when injected into Limulus photoreceptor cells, suggesting that it may have a signaling role separate from that of IP\(_3\) in some cells.\(^{139}\)

**The Cytosolic Free Ca\(^{2+}\) Transient**

The typical agonist-induced effect on cytosolic free Ca\(^{2+}\) is an initial rapid increase followed by a gradual fall over the ensuing several minutes. This is illustrated in Figure 4 for the effect of a maximal concentration of vasopressin in isolated hepatocytes loaded with the fluorescent Ca\(^{2+}\) indicator Quin 2.\(^{140}\) The initial increase of Ca\(^{2+}\) is entirely due to mobilization of Ca\(^{2+}\) from the IP\(_3\)-sensitive Ca\(^{2+}\) pool, since it is unaffected by the removal of extracellular Ca\(^{2+}\) by EGTA immediately before the addition of hormone.\(^{105,141,142}\) The fall of cytosolic free Ca\(^{2+}\) from its peak value coincides with a net efflux from the cell, which can occur against the physiological gradient of Ca\(^{2+}\) across the plasma membrane of approximately 10\(^5\). The net loss of Ca\(^{2+}\) from the cell is less than the maximal amount that can be mobilized by hormone, and it is probable that part of the fall in cytosolic free Ca\(^{2+}\) is associated with an increased uptake into cellular organelles, notably the mitochondria, where it activates pyruvate and \(\alpha\)-ke-
The transient Ca\(^{2+}\) response illustrated in Figure 4 appears, in principle, to be typical of those observed in many cells after addition of Ca\(^{2+}\)-mobilizing agonists, with the first (peak) phase being essentially independent of extracellular Ca\(^{2+}\) and the second (maintenance) phase being Ca\(^{2+}\)-dependent. It may be noted that in instances in which the peak of the Ca\(^{2+}\) response is depressed in low-Ca\(^{2+}\) medium, relative to the control, this is probably caused by net Ca\(^{2+}\) efflux from the cell and loss of Ca\(^{2+}\) from the IP\(_3\)-sensitive Ca\(^{2+}\) pool before addition of the agonist. \(^{11-14}\) The inability of agonists to cause an increase of cytosolic free Ca\(^{2+}\) in calcium-depleted cells is not due to an uncoupling of the receptor from phospholipase C, as shown recently with PC12 cells. \(^{142}\) After the cells had been treated with ionomycin and incubated in Ca\(^{2+}\)-free medium, the addition of carbachol caused an increase of IP\(_3\) but no increase of cytosolic free Ca\(^{2+}\), as monitored with Quin 2. With some cells and agonists (e.g., stimulation of pituitary GH\(_3\) cells by thyrotropin-releasing hormone) there is a secondary extracellular Ca\(^{2+}\)-dependent slow rise of cytosolic free Ca\(^{2+}\) after the initial peak, rather than a steady state plateau value, because of a net entry of Ca\(^{2+}\) by a voltage-dependent Ca\(^{2+}\) channel. \(^{146-150}\) Cyclic IP\(_3\), or IP\(_3\) mediation of an agonist-induced effect on plasma membrane Ca\(^{2+}\) flux is an intriguing possibility that remains to be explored. \(^{131}\) In recent experiments with perfused rat livers where net Ca\(^{2+}\) flux changes across the plasma membrane induced by Ca\(^{2+}\)-mobilizing hormones were measured with a Ca\(^{2+}\) electrode placed in the effluent fluid, vasopressin and angiotensin II stimulated net Ca\(^{2+}\) uptake after an initial net Ca\(^{2+}\) efflux phase, whereas phenylephrine caused only a net Ca\(^{2+}\) efflux while the receptors were still occupied by the hormones. \(^{150}\) It is possible that these differences may be related to quantitative differences between the agonist-induced accumulations of inositol lipid metabolites (e.g., of IP\(_4\) or diacylglycerol).

### Plasma Membrane Ca\(^{2+}\) Fluxes

As noted in the previous section, the falling phase of the agonist-induced response of cytosolic free Ca\(^{2+}\) is caused by a net efflux of Ca\(^{2+}\) from the cell. Transport of Ca\(^{2+}\) out of the cell probably occurs through plasma membrane Ca\(^{2+}\)-ATPase in response to the increase in cytosolic free Ca\(^{2+}\). In several different cell types (e.g., liver \(^{111}\) and neutrophils \(^{152}\)) the onset of this net Ca\(^{2+}\) efflux is delayed relative to the rise of cytosolic free Ca\(^{2+}\). The efflux is caused by an inhibition of Ca\(^{2+}\)-ATPase, which persists after isolation of plasma membrane from hormone-treated tissue. \(^{113,114}\) The mechanism responsible for this inhibition has not been elucidated, but it may be caused by the hormone-induced decrease of the polyphosphoinositides in the plasma membrane, since these phospholipids have been found to increase the activity of purified erythrocyte Ca\(^{2+}\)-ATPase reconstituted into phosphatidyicholine liposomes. \(^{153}\) A relative inhibition of Ca\(^{2+}\) efflux from the cell may serve an important function in allowing the cells to retain the Ca\(^{2+}\) released from the IP\(_3\)-sensitive Ca\(^{2+}\) pool during the early phase of agonist stimulation, so that the Ca\(^{2+}\)-mediated functional effects (e.g., activation of protein kinases) can be fully expressed. Thus, once Ca\(^{2+}\)-mobilizing hormones have been added, phosphorylase a activity in liver remains elevated long after the decline of cytosolic free Ca\(^{2+}\) from its peak value (see Figure 4).

Both in the resting cell and in the stimulated cell in the steady state with respect to Ca\(^{2+}\), there is an exchange of Ca\(^{2+}\) across the plasma membrane. Cal-
cium-mobilizing agonists are known to increase this plasma membrane Ca\(^{2+}\) permeability.\(^ {7,9,12,156}\) Calcium enters the cell by a number of different pathways, depending on the cell type. The best-understood mechanism is voltage-dependent Ca\(^{2+}\) gating, in which Ca\(^{2+}\) entry occurs during membrane depolarization and is highly sensitive to inhibition by Ca\(^{2+}\) channel-blocking agents, such as verapamil or nifedipine. This mechanism appears to be responsible for agonist-stimulated Ca\(^{2+}\) entry in some secretory cells, such as pancreatic islets and GH\(_3\) cells, as previously mentioned. A different but poorly understood mechanism has been described by the term receptor-mediated Ca\(^{2+}\) channel because of its dependence on agonist stimulation and its relative or selective sensitivity to different types of Ca\(^{2+}\) channel blockers.\(^ {157,158} \) This process may not involve an electrogentic entry of Ca\(^{2+}\) down its concentration gradient through a Ca\(^{2+}\) channel but instead may be mediated by an exchange transport with H\(^{+}\) or Na\(^{+}\). A Ca\(^{2+}\)-Na\(^{+}\) exchange with a stoichiometry of approximately 1:3 has been well documented in the plasma membrane of cardiac, nerve, and smooth muscle tissue,\(^ {159} \) and recently in pancreatic acinar cells,\(^ {160} \) and it might be involved in hormone-stimulated Ca\(^{2+}\) entry into cells such as those in the liver, which have a low resting membrane potential. It is also not clear whether a postulated Ca\(^{2+}\)-H\(^{+}\) exchange exists as such in the plasma membrane or whether this is represented by the combined effects of Ca\(^{2+}\)-Na\(^{+}\) and Na\(^{+}\)-H\(^{+}\) exchange reactions.\(^ {161} \) Na\(^{-}\)-H\(^{+}\) exchange, which has been shown to be present in a number of different cell types,\(^ {162} \) is of interest because it is stimulated by activation of protein kinase C. An increased intracellular Na\(^{+}\) concentration may make Ca\(^{2+}\) entry by a Ca\(^{2+}\)-Na\(^{+}\) exchange energetically favorable. Whether such a mechanism accounts for agonist-stimulated Ca\(^{2+}\) entry into cells has not yet been ascertained, but it is of interest that an increased Ca\(^{2+}\) influx by way of a Ca\(^{2+}\)-Na\(^{+}\) exchange upon removal of extracellular Na\(^{+}\) has recently been demonstrated in monkey kidney cells.\(^ {163} \) Because the specific mechanisms of receptor-mediated Ca\(^{2+}\) entry into cells remain largely unidentified, various studies with many cell types indicate that sustained functional responses to Ca\(^{2+}\)-mobilizing agonists are dependent on an increased rate of Ca\(^{2+}\) entry into the cell.

**Functional Roles of Protein Kinase C Activation**

In the resting cell, protein kinase C is mainly found in the soluble fraction in an inactive state. Enzyme activity requires an acidic phospholipid, with phosphatidylyserine being more effective than PI or phosphatidylethanolamine. Activation under agonist stimulation is associated with the binding of protein kinase C to the plasma membrane, promoted by an increased diacylglycerol concentration in the membrane. Diacylglycerol decreases the Ca\(^{2+}\) requirement for the enzyme, so that it is active at resting cellular Ca\(^{2+}\) concentrations.\(^ {15,163-165} \) From the kinetic behavior of the isolated enzyme, a further increase in activity may occur as a consequence of an increased Ca\(^{2+}\) concentration, although this point has not been demonstrated in studies with intact cells. As far as it is known, expression of the effects of protein kinase C activation is mediated only when the enzyme is bound to the plasma membrane. Although 1,2-diacylglycerol is the physiological activator of protein kinase C, it is also directly activated by various tumor-promoting agents, of which phorbol myristate acetate (PMA) has been most commonly used. However, unlike diacylglycerol, which is regulated in the cell by a number of enzymes that convert it to products having little effect on protein kinase C activity, phorbol esters produce a permanent activation of the enzyme. Thus, effects induced by the addition of PMA to cells do not necessarily mimic the effects induced by hormone activation of protein kinase C through diacylglycerol production.

From the results of many studies using PMA with a variety of tissues, the following general conclusions can be drawn concerning functional effects mediated by protein kinase C-induced protein phosphorylation. 1) It appears to have no effect by itself on inositol lipid breakdown and Ca\(^{2+}\) mobilization. 2) In many secretory cells activation of protein kinase C by PMA acts synergistically with increased cytosolic free Ca\(^{2+}\) to produce a sustained functional response. 3) In many cells PMA prevents or attenuates the Ca\(^{2+}\)-mobilizing response to certain (but not all) agonists at a step before activation of phospholipase C. 4) The levels of PIP and PIP\(_2\) are increased in some cells, possibly by a stimulation of the activity of PI (or PIP) kinases. 5) The plasma membrane Na\(^{-}\)-H\(^{+}\) exchange is promoted in a variety of cells with a small increase of cytosolic pH. 6) Cell growth and differentiation are promoted under suitable conditions.

Addition of a Ca\(^{2+}\)-mobilizing hormone or suitable agonist to secretory cells produces a sustained functional effect, provided that the cell surface receptors remain activated. However, it has been observed that when the cytosolic free Ca\(^{2+}\) concentration alone is increased (e.g., by addition of the Ca\(^{2+}\) ionophore A23187 or by K\(^{+}\) depolarization), a transient or incomplete secretory response is obtained. Likewise, addition of PMA or synthetic diacylglycerols alone produces a slow and submaximal secretory response. However, an increase of cytosolic free Ca\(^{2+}\) combined with activation of protein kinase C causes an enhanced and sustained secretory response equivalent to that produced by agonist stimulation. Such a synergism between Ca\(^{2+}\) and PMA has been observed for serotonin secretion from platelets,\(^ {166,169} \) insulin secretion from pancreatic islet cells,\(^ {170,171} \) aldosterone secretion from adrenal cells,\(^ {172} \) prolactin secretion from pituitary cells,\(^ {173-175} \) amylase secretion from pancreatic acinar cells,\(^ {176} \) acetylcholine release from ileal nerve endings,\(^ {177} \) and enzyme secretion from parotid glands.\(^ {178} \) Since Ca\(^{2+}\)-calmodulin-activated protein kinases and protein kinase C promote the phosphorylation of different sets of target proteins, it is apparent that the integrated cellular response requires the cooperative effects of both the IP\(_3\) and the diacylglycerol second-messenger systems. However, how these pro-
proteins interact in the various stages of the overall secretory response has not been ascertained. Although it is evident that an increase of Ca\(^{2+}\) alone can trigger secretory activity and is essential for the rapid onset of secretion, it is not clear whether sustained secretion requires a small steady state elevation of the cytosolic free Ca\(^{2+}\) as well as an activation of protein kinase C.\(^7, 14, 154, 179\)

Not all cells show a synergism between Ca\(^{2+}\)-mediated and protein kinase C-mediated effects, indicating separate or parallel functions of the two messenger systems. Thus, recent studies in hepatocytes have shown that PMA has no synergistic effect with Ca\(^{2+}\) ionophores in increasing phosphorylase \(\alpha\) levels and cytosolic free Ca\(^{2+}\).\(^180, 181\) However, PMA has also been shown to inhibit \(\alpha\)\(_R\)-adrenergic-induced and, to a lesser extent, vasopressin-induced Ca\(^{2+}\) mobilization responses in liver.\(^180, 181\) Chemotactic factor-induced or concanavalin A-induced Ca\(^{2+}\) mobilization in neutrophils,\(^182, 183\) and muscarinic cholinergic effects in transformed smooth muscle (PC12) cells,\(^184\) as well as agonist-induced formation of inositol phosphates or Ca\(^{2+}\) mobilization in platelets,\(^119, 185, 186\) hippocampal slices,\(^187\) astrocytoma cells,\(^188\) and pituitary GH\(_3\) cells.\(^189\) In intact smooth muscle, PMA had either synergistic effects (rat vas deferens and canine basilar artery) or inhibitory effects (guinea pig ileum and rat uterus) on neurotransmitter actions governing contractility.\(^190\) Although PMA had inhibitory effects on angiotensin II stimulation of IP\(_3\) formation and Ca\(^{2+}\) mobilization in cultured thoracic aorta smooth muscle cells,\(^191\) these inhibitory effects of PMA on cell function mediated by certain agonists appear to be somewhat anomalous, since the agonists themselves cause an activation of protein kinase C. The degree of activation of protein kinase C is probably less severe in these enzymes than in those in which the enzyme is artificially activated by PMA, and this distinction may also influence the number of protein kinase C target proteins that become phosphorylated (R.H. Cooper, R. Johanson, and J.R. Williamson, submitted for publication, 1986). In studies with hepatocytes no evidence was obtained that PMA affected cytosolic free Ca\(^{2+}\), Ca\(^{2+}\) influx, or Ca\(^{2+}\) efflux.\(^180\) However, studies with neutrophils\(^182\) and GH\(_3\) cells\(^189\) indicate that PMA stimulates Ca\(^{2+}\) efflux from the cell, although it has not been demonstrated that the plasma membrane Ca\(^{2+}\)-ATPase is phosphorylated by protein kinase C or regulated by a phosphorylated protein.

The effect of PMA is mediated partly at the level of the receptor itself, since agonist binding is inhibited. This may be a consequence of phosphorylation of the receptor by protein kinase C, since receptors for somatotropin, insulin, epidermal growth factor, transferrin, and histone H\(_4\) have been shown to be endogenous substrates for the enzymes.\(^167\) Recent studies with a cell line (DDT, MF-2) derived from hamster vas deferens smooth muscle have shown that PMA results in a decreased ability of \(\alpha\)\(_R\)-adrenergic agonists to stimulate PI turnover, in association with a marked increase in the phosphorylation of the \(\alpha\)\(_R\)-receptor.\(^192\) Further studies are needed to determine the extent to which receptor phosphorylation leads to an uncoupling of signal transduction from the receptor to the putative GTP-binding protein required for activation of phospholipase C, as compared with effects at the postreceptor level, such as phosphorylation of the GTP-binding protein itself. It appears likely, however, that protein kinase C activation is involved in receptor internalization or desensitization as a general mechanism for termination of agonist-mediated transduction processes.\(^193\)

In contrast, the apparent effect of protein kinase C in increasing PI and PIP kinase activation may provide positive feedback for agonist stimulation, since these enzymes are primarily responsible for maintaining the phosphoinositol levels as precursor substrates for generation of IP\(_3\) and diacylglycerol. So far, the phenomenon has been reported only in platelets\(^186, 194, 195\) and thymocytes,\(^196\) and the mechanism by which inositol lipid kinases are stimulated has not been elucidated. Furthermore, it has not been demonstrated that an increase in PIP\(_2\) levels in cells has any effect on the rate of formation of the second messengers. The main effect of a stimulation of inositol lipid kinase may be to ensure a prolonged response to the agonist in some cells during the phase of active growth. The deleterious effects of tumor-promoting agents, on the other hand, may be accounted for by an overstimulation of protein kinase C and deranged cell growth.\(^193\)

Various growth factors, which were previously shown to stimulate inositol lipid metabolism,\(^197\) cause an elevation of cytosolic free Ca\(^{2+}\),\(^198\) which is thought to be a requirement for cell proliferation.\(^11, 193\) However, in most cells an increase of Ca\(^{2+}\) alone is an insufficient signal for cell growth, indicating that other factors must also be involved. Of particular interest is the demonstration that phorbol esters promote Na\(^+\)-H\(^+\) exchange across the plasma membrane, with a consequent low alkalization of the cell interior\(^199-205\) — an effect that is thought to be important for cell proliferation. It has been shown that the stimulating effect of growth factors on the Na\(^+\)-H\(^+\) exchange in rat myoblasts is due to an increase of its maximal velocity and affinity for protons, with PMA mimicking the latter effect.\(^206\) The relative concentrations of Na\(^+\) and H\(^+\) across the plasma membrane make transport thermodynamically favorable in the direction of Na\(^+\) influx and H\(^+\) efflux. Cells normally buffer the intracellular pH quite effectively, but a potentially important secondary consequence of an increased intracellular Na\(^+\) concentration is a stimulation of the Na\(^+\), K\(^+\)-ATPase or Ca\(^{2+}\)-Na\(^+\) exchange.\(^207\) Alterations of these ion transport systems not only affect the electrical potential across the plasma membrane but may also contribute to regulation of the cytosolic free Ca\(^{2+}\) concentration, as discussed above.

**Conclusions**

A large number of recent studies with many different cell types have established several important features of the role of inositol lipids in signal transduc-
tion. Studies of the agonist-induced breakdown of polyphosphoinositides, and particularly of changes in inositol phosphate products in stimulated cells, provide evidence that IP₃ has a central role in Ca²⁺ mobilization. A number of long-debated issues have been resolved by measurements of changes in cytosolic free Ca²⁺ with the fluorescent Ca²⁺ indicator Quin 2 and the addition of IP₃ to permeabilized cells and subcellular organelles, together with the direct injection of IP₃ into large cells. Thus, agonists that exert their effects on cell function by Ca²⁺-dependent rather than cAMP-dependent mechanisms elicit an increase of cytosolic free Ca²⁺ by an IP₃-mediated mobilization of calcium from intracellular stores instead of by direct entry of Ca²⁺ across the plasma membrane. Receptor-dependent increases of plasma membrane Ca²⁺ permeability, however, are obligatory for sustained functional effects. The source of the intracellular calcium has been identified as a subpopulation of the endoplasmic reticulum rather than the mitochondria or plasma membrane, as previously suggested. The location of the specialized regions of the endoplasmic reticulum within the cell, the nature of the IP₃ receptor, its possible regulation, and the mechanism of IP₃-induced Ca²⁺ release remain to be elucidated. The recent discoveries that cyclic IP₃ and IP₄ are rapidly produced in cells after agonist stimulation raise the possibility that these compounds may have additional roles in regulating stimulus-response coupling.

Much effort is currently being devoted to the question of how receptor occupancy is coupled with phospholipase C to initiate inositol lipid breakdown. Many different studies indicate the involvement of a GTP-binding protein in signal transduction. In neutrophils, mast cells, and platelets, agonist-stimulated breakdown of inositol lipids and Ca²⁺ mobilization are inhibited by pertussis toxin, in conjunction with ADP ribosylation of a protein in the plasma membrane with characteristics similar to Gₛ, the inhibitory GTP-binding protein that is coupled to adenylate cyclase. In many other cell types, however, agonist-induced Ca²⁺ mobilization is not susceptible to inhibition by pertussis toxin. At present, it is not clear whether Gₛ has a bifunctional role in some cells in exerting both an inhibitory effect on adenylate cyclase and a stimulatory effect on phospholipase C, depending on the type of receptor occupied by agonists, or whether an unidentified GTP-binding protein that has differential specificity to inactivation by pertussis toxin uniquely activates phospholipase C. Further developments in this area will also involve elucidation of the regulation of the coupling functions of GTP-binding proteins by protein phosphorylation.

Agonist-induced hydrolysis of inositol lipids to produce IP₃ as the Ca²⁺-mobilizing second messenger is necessarily coupled with formation of diacylglycerol as the second cleavage product. This compound is a physiological activator of protein kinase C, which is the receptor for tumor-promoting phorbol esters; both agents induce the binding of soluble protein kinase C to plasma membrane and cause activation by lowering the Ca²⁺ concentration required for enzyme activity. The physiological effects of activation of protein kinase C by diacylglycerol have not been fully clarified, and the identity and function of many of the target proteins phosphorylated by protein kinase C under physiological conditions of agonist stimulation are not known. Activation of protein kinase C by phorbol esters produces a permanent, unphysiological activation of the enzyme; hence, the degree to which phorbol ester-mediated activation of protein kinase C mimics activation of the enzyme under physiological conditions remains uncertain. Several aspects of cell function are modified by phorbol ester-induced activation of protein kinase C, and effects mediated by protein phosphorylations have been implicated in the maintenance of a sustained secretory response, receptor desensitization, and cell proliferation. One of the most exciting challenges for future research is to evaluate how different receptor-generated signaling systems that activate a variety of protein kinases and phosphatases interact with each other to modulate cell function, growth, and differentiation.

References

17. Siess W, Siegel FL, Lapetina EG. Arachidonic acid stimu-
lates the formation of diacylglycerol and phosphatidic acid in human platelets. J Biol Chem 1983;258:11236-11242


19. Rittenhouse SE. Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclooxygenase products and ADP. Biochem J 1984;222:103-110


24. Heyworth CM, Whetton AD, Wong S, Martin BR, Houslay MD. Activation of membrane responses by insulin inhibits the cholera-toxin-catalyzed ribosylation of a Mr 25,000 protein in rat liver plasma membranes. Biochem J 1985;229:593-603


35. Pfaffinger PJ, Martin JM, Hunter DD, Nathanson NM, Hille B. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature 1985;317:536-538


46. Compert BD. Involvement of guanine nucleotide binding protein in the gating of Ca2+ by receptors. Nature 1983;306:64-66

47. Haslam RJ, Davidson MML. Guanine nucleotides decrease the free Ca2+ required for secretion of serotonin from permeabilized blood platelets: evidence of a role for GTP-binding protein. FEBS Lett 1984;174:90-95


54. Lucas DO, Bajajieh SM, Kowalchyk JA, Martin TJF. Direct stimulation by thyrotropin-releasing hormone (TRH) of polyphosphoinositide hydrolysis in GH3 cell membranes by a guanine nucleotide-modulating mechanism. Biochem Biophys Res Commun 1983;122:721-728


58. Molski TP, Naccache PH, Marsh ML, Kermode J, Becker EL, Sha'af RI. Pertussis toxin inhibits the rise in the intracellular concentration of free calcium that is induced by chemo- tactic factors in rabbit neutrophils: possible role of the "G-proteins" in calcium mobilization. Biochem Biophys Res Commun 1984;124:644-650

59. Volpi M, Naccache PH, Molski TP, et al. Pertussis toxin...
inhibits \(\text{Met-Leu-Phe}\) but not phorbol ester-stimulated changes in rabbit neutrophils: role of G proteins in excitation response coupling. Proc Natl Acad Sci USA 1985;82:2708-2712


63. Lad PM, Olson CV, Smiley PA. Association of the N-formyl-Met-Leu-Phe receptor in human neutrophils with a GTP-binding protein sensitive to pertussis toxin. Proc Natl Acad Sci USA 1985;82:869-873


70. Evans T, Martin MW, Hughes AR, Harden TK. Guanine nucleotide sensitive, high-affinity binding of carbachol to muscarinic cholinergic receptors of 132NI astrocytoma cells is insensitive to pertussis toxin. Mol Pharmacol 1985;27:32-37


75. Lynch CJ, Ppivec V, Blackmore PF, Epton JH. Effect of islet-activating pertussis toxin on the binding characteristics of \(Ca^2+\)-mobilizing hormones and on agonist activation of phospholipase in hepatocytes. Mol Pharmacol 1986;29:196-203


82. Joseph SK, Williams RJ. Subcellular localization and some properties of the enzymes hydrolysing inositol polyphosphates in rat liver. FEBS Lett 1985;180:150-154


96. Akhtar RA, Abdel-Latif AA. Carbachol causes rapid phosphodiesterase cleavage of phosphatidyl-myo-inositol 4,5-bisphosphate and accumulation of inositol phosphates in rabbit iris smooth muscle; prazosin inhibits noradrenaline- and io-
II-154

1985 BLOOD PRESSURE COUNCIL

phone A23187-stimulated accumulation of inositol phosphates. Biochem J 1984;224:291-300


98. Bocckino SB, Blackmore PF, Exton JH. Stimulation of 1,2-diacylglycerol accumulation in hepatocytes by vasopressin, epinephrine and angiotensin II. J Biol Chem 1985;260:14201-14207


103. Scherer NM, Ferguson JE. Inositol 1,4,5-trisphosphate is not effective in releasing calcium from skeletal sarcoplasmic reticulum. Biochem Biophys Res Commun 1985;128;1066-1070

104. Van Rooijen LAA, Rossowska M, Bazan NG. Inhibition of phosphatidylinositol-4-phosphate kinase by its product phosphatidylinositol-4,5-bisphosphate. Biochem Biophys Res Commun 1985;126:150-155


116. Dawson AP, Irvine RF. Inositol 1,4,5-trisphosphate promotes Ca²⁺ release from microsomal fractions of rat liver. Biochem Biophys Res Commun 1984;120:858-864


118. Dawson AP, GTP enhances inositol trisphosphate-stimulated Ca²⁺ release from rat liver microsomes. FEBS Lett 1985;185:147-150


120. Prenki M, Corkey BE, Matschinsky FM. Inositol 1,4,5-trisphosphate and the endoplasmic reticulum Ca²⁺ cycle of a rat insulinoma cell line. J Biol Chem 1985;260:9185-9190


129. Batty IR, Nahorski SR, Irvine RF. Rapid formation of inositol 1,4,5-trisphosphate following muscarinic receptor activation in rat cardiac myocytes and sarcoplasmic reticulum. FEBS Lett 1985;198:328-332

130. Harada M, Sasa M, Hasegawa Y, Ishimura Y, Hara H. Inhibition of Ca²⁺ release from microsomal fractions of rat liver. Biochem Biophys Res Commun 1984;128;1066-1070


132. Dawson AP, Irvine RF. Inositol 1,4,5-trisphosphate promotes Ca²⁺ release from microsomal fractions of rat liver. Biochem Biophys Res Commun 1984;120:858-864

133. Batty IR, Nahorski SR, Irvine RF. Rapid formation of inositol 1,4,5-trisphosphate following muscarinic receptor activation in rat cardiac myocytes and sarcoplasmic reticulum. FEBS Lett 1985;198:328-332


135. Chakraborti S, Biswas BB. Evidence for the existence of a


146. Gershengorn MC, Thaw C. Calcium influx is not required for calcium influx in hepatocytes following α1-adrenergic induced changes in intracellular [Ca2+] measured by microspectrofluorometry on individual Quin 2-loaded hepatocytes. J Biol Chem 1984;259:14448–14457


152. Martin TFJ, Kowalchyk JA. Evidence for the role of calcium and diacylglycerol as dual second messengers in thyrotropin-
releasing hormone action: involvement of Ca\(^{2+}\). Endocrinology 1984;115:1527–1536


186. Watson SP, Lapetina EG. 1,2-Diacylglycerol and phorbol ester inhibit agonist-induced formation of inositol phosphates in human platelets: possible implications for negative feedback regulation of inositol phospholipid hydrolysis. Proc Natl Acad Sci USA 1985;82:2623–2627


199. Rosoff PM, Stein LF, Cantley LC. Phorbol esters induce differentiation in a pre-B-lymphocyte cell line by enhancing Na\(^+\)/H\(^+\) exchange. J Biol Chem 1984;259:7056–7060


Role of inositol lipid breakdown in the generation of intracellular signals. State of the art lecture.

J R Williamson

Hypertension. 1986;8:II140
doi: 10.1161/01.HYP.8.6_Pt_2.II140

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1986 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/8/6_Pt_2/II140

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/