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 Ca2+ 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 Ca2+-binding proteins. Until recently, however, the source of the Ca2+, the amount and kinetics of the Ca2+ changes, and the mechanism of hormone-stimulated cellular Ca2+ 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 monoacylglyceride 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\(_i\) 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-bisphosphate (IP\(_2\)), 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 adenylyl cyclase.

Receptor-mediated stimulation (e.g., by β-adrenergic agonists) or inhibition (e.g., by α1-adrenergic agonists or muscarinic agonists acting on M2 receptors) of adenylyl 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 Gs and Gi, the α-subunits are different (relative molecular weight, 52,000 or 45,000 for αs and 41,000 for αi), whereas the βγ-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 adenylyl cyclase occurs when αs-GTP binds to the enzyme. This stimulatory signal is terminated by intrinsic GTPase activity of the subunit, followed by reassociation of αs-GDP with the βγ-subunits. Inhibition of activated adenylyl 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 αi-subunit as a consequence of an increased availability of βγ-subunits upon dissociation of Gi.

In addition to Gs and Gi, 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 Gs and Gi. The function of Gq has not yet been elucidated, but recent voltage-clamp studies with atrial cells suggest
plasma membrane preparations from neutrophils, \(^{39}\) blowfly salivary glands, \(^{39}\) polymorphonuclear leukocytes, \(^{41\text{-}43}\) hepatocytes, \(^{52,54}\) GH, pituitary cells, \(^{54}\) arterial smooth muscle \(^{55}\) and cerebral cortical membranes, \(^{56}\) 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) \(\alpha\)-subunit of GTP-binding protein may be to decrease the \(Ca^{2+}\) requirement for activation of phospholipase C. \(^{56}\)

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, \(^{51,53\text{-}63}\) mast cells, \(^{64}\) human leukemic HL60 cells, \(^{65,66}\) and the hybrid cell line WBC-264-9C, \(^{67}\) chemotactic peptide-induced interactions with inositol lipid metabolism, arachidonic acid release, \(O_2^-\) generation, \(Ca^{2+}\) 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 adenylate cyclase in neutrophil plasma membranes, with the effect being abolished by pertussis toxin pretreatment. \(^{68}\) 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 \(G_i\). The involvement of \(G_i\) in coupling the chemotactic peptide receptor in neutrophils was substantiated by further studies showing that the addition of \(G_i\) (isolated from brain) to pertussis toxin-pretreated membranes was able to restore fMet-Leu-Phe binding affinity and fMet-Leu-Phe-induced GTPase activity, which had been inhibited by the pertussis toxin treatment. \(^{69}\) The conclusion reached from these studies was that chemotactic peptide receptors in neutrophils and similar cells are coupled to \(G_i\) and that this \(G_i\) protein has a bifunctional role in inhibiting adenylate cyclase and activating inositol lipid metabolism. In other cell types, however, pertussis toxin treatment apparently has no effect in inhibiting the actions of \(Ca^{2+}\)-mobilizing hormones. Thus, pertussis toxin pretreatment had no observed effect on the binding of \(\alpha\)-adrenergic ligands to rat kidney cortex membranes \(^{70}\) or of muscarinic ligands to receptors of astrocytoma cells \(^{70}\) in the absence or presence of guanine nucleotides. More directly, it has been shown that treatment of astrocytoma and chick heart cells, \(^{71}\) 3T3 fibroblasts, \(^{72}\) pituitary GH, cells, \(^{73}\) and hepatocytes \(^{74,75}\) with concentrations of pertussis toxin that apparently cause complete ADP ribosylation of \(G_i\) and prevention of its inhibitory effects on adenylate cyclase does not prevent agonist-induced increases of polyphosphoinositide breakdown or \(Ca^{2+}\) mobilization. However, pertussis toxin pretreatment of rats has been shown to prevent \(\alpha\)-adrenergic stimulation of PI turnover in subsequently isolated adipocytes. \(^{76}\) In contrast, pertussis toxin had no observed effect on \(\alpha\)-adrenergic stimulation of respiration in brown adipocytes. \(^{77}\) In these studies, pertussis toxin augmented \(\beta\)-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 \(G_i\) on adenylate 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 \(\alpha\)-subunit of \(G_i\). In other cells, however, the \(G\) protein involved in \(Ca^{2+}\) mobilization appears not to be affected by pertussis toxin, suggesting that it is distinct from \(G_i\). 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, yet unidentified, \(G\) binding protein that differs from \(G_i\) 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 \(Ca^{2+}\), unlike that of chemotractant peptides, is not sensitive to inhibition by pertussis toxin. \(^{51}\) It is possible, therefore, that specific receptors in certain cells form a complex with the phospholipase C–coupled \(G\) protein to make it susceptible 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 \([^{3}H]\)inositol and \([^{32}P]\)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. \(^{79}\) The phosphomonoesterase-
ases selectively remove phosphate from the fourth and fifth positions of the inositol ring and convert PIP₃ to PIP₂, 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 IP₃, IP₂, and IP₃, from PI, PIP, and PIP₂, respectively (see Figure 2). Various phosphatases are responsible for the successive hydrolysis of IP₃ to IP₂, IP₂ to IP₁, and IP₁ to inositol. The IP₁ and IP₂ phosphatases, but not the IP₃ phosphatase, are sensitive to inhibition by Li⁺, and all the phosphatases require Mg²⁺ 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 [³P] from [¹⁴C]adenosine 5'-triphosphate (ATP) into PA and subsequently into PI provided the basis for measurements of PI turnover in the earlier literature.

Since diacylglycerol and IP₃ have separate and distinct roles as signal generators, a question of fundamental importance concerns the regulation of the substrate specificity of phospholipase C during hormone stimulation. Studies with intact cells have shown that hydrolysis of PIP and PIP₂ precedes that of PI. In addition, a number of kinetic studies with different tissues have shown that IP₃ is produced before IP₂ and simultaneously with or in some cells before IP₁.

Consequently, it has been postulated that in the intact cell IP₃ and IP₂ are produced primarily from hydrolysis of IP₃, with the PIP and PIP₂ pools being replenished by the inositol lipid kinases. This hypothesis is probably not correct since it would imply that IP₁ and diacylglycerol are produced in stoichiometrically equal amounts, which is not the case. However, this issue has not yet been completely resolved because of the technical difficulty of measuring unidirectional fluxes through the individual inositol lipid kinases and phosphomonoesterase steps, as well as the absence of selective inhibitors of the phosphatases that hydrolyze PI₃ and IP₃.

A related issue concerns the dependence of phospholipase C on Ca²⁺ for its different inositol lipid substrates. In contrast to earlier concepts, it is probable that a single phosphodiesterase in the plasma membrane is responsible for the agonist-induced breakdown of polyphosphoinositides. In rat liver this breakdown has been shown to occur predominantly in the plasma membrane. Studies with purified phospholipase C reconstituted along with radiolabeled inositol lipid substrates into phospholipid vesicles have shown that whereas Ca²⁺ stimulated the breakdown of all three inositol lipids, only the polyphosphoinositides were hydrolyzed in the presence of ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Hydrolysis of PI is completely dependent on Ca²⁺, with a Michaelis constant of 1 to 2 μM. These studies with the isolated enzyme are in accordance with a large number of studies with intact cells showing that although the agonist-induced breakdown of PIP and PIP₂ is dependent on Ca²⁺, PI hydrolysis and turnover can be stimulated by an increase of the intracellular Ca²⁺ concentration induced by the Ca²⁺ ionophore A23187. However, in the GH₃ pituitary cell, the Ca²⁺ ionophore causes an enhanced breakdown of both PI and PIP, with production of IP₃, IP₂, diacylglycerol, and arachidonic acid but no IP₁, in contrast to the effects observed in studies with thyrotropin-releasing hormone, in which the formation of IP accounts for the hormone-induced increase of cytosolic free Ca²⁺. Thus, in some cells the breakdown of PI as well as PI may be sensitive to Ca²⁺. In other preparations, such as brain synaptosomes and iris smooth muscle, addition of A23187 in the presence of extracellular Ca²⁺ has been shown to cause a breakdown of each of the inositol lipids, raising the question of whether hormone-stimulated hydrolysis of the polyphosphoinositides was secondary to an elevation of cytosolic Ca²⁺ resulting from increased entry of Ca²⁺ into the cell. In the case of iris smooth muscle, it was later demonstrated that the α₁-adrenergic antagonist prazosin prevented increases of IP₃ induced by A23187, suggesting that the breakdown of PI 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 Ca²⁺ activation of phospholipase A₂, 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 IP₃-induced Ca²⁺ 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 stimulate diacylglycerol.

The relative amounts of diacylglycerol and IP₃ 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 IP₃ to elicit mobilization of Ca²⁺ but not enough diacylglycerol to activate protein kinase C fully. Expression of the diacylglycerol signaling role, therefore, may require simultaneous hydrolysis of PIP and particularly PI. Conversely, it is possible that some tissues, such as cardiac muscle, may lack the Ca²⁺ mobilization branch of the signaling system because of the absence or low sensitivity of the IP₃ receptor in the sarcoplasmic reticulum while retaining the diacylglycerol-activated protein kinase C branch. Vascular smooth muscle and frog skel-
terases. In the kinetic study with platelets, however, it is required before the regulation of PIP and PIP 2 to PIP by PI kinase. Further studies with different enzymes can be defined in more detail.

Product inhibition, as demonstrated for PIP kinase, may also account for an increase of flux through the PIP kinase. Kinetic studies using [32P]phosphate labeling with platelets have in fact demonstrated an increase of flux through PIP kinase in conjunction with a thrombin-stimulated breakdown of PIP 2. It has been suggested that regulation of PI and PIP kinase activities may be mediated by activation of protein kinase C (see below). Relief of the inositol lipid kinases from product inhibition, as demonstrated for PIP 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 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 by PI kinase. Further studies with different cells are required before the regulation of PIP and PIP 2 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 have a direct signaling role in the mobilization of intracellular Ca 2+, 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 be secondary to the rise of cytosolic free Ca 2+. 2) PIP 2 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 2, 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 and Xenopus eggs. Studies with hepatocytes have shown that the rate of breakdown of IP 3 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+. Similarly, injection of IP 3 into single cells has now shown that injection of IP 3 into photoreceptors, Limulus eggs. Studies with hepatocytes have shown that the rate of breakdown of IP 3 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. 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 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.

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Effects of inositol-1,4,5-trisphosphate (IP3) on Ca2+ release in saponin-permeabilized hepatocytes. Permeabilized cells were incubated in a buffered KCl medium containing 3 mM MgATP. IP3 was added to give a concentration of 0.5 μM, followed by the addition of 2 mM ATP. Changes in medium free Ca2+ were measured using 75 μM Quin 2. The results are shown in Panel A. Panel B shows the results of experiments with cells incubated in a similar medium (minus Quin 2) before the addition of saponin. IP3 infusion was continued as shown by the horizontal bar. Medium free Ca2+ was measured with a Ca2+ electrode.

The transient rather than sustained release of Ca2+ upon addition of IP3 to permeabilized cells (see Figure 3A) or partially purified endoplasmic reticulum preparations can be accounted for by the presence of an IP3 phosphatase, which causes hydrolysis of IP3 to the inactive product IP2. The problem of continuous disappearance of the added IP3 has been overcome by infusion of IP3 to permeabilized cell preparations, which causes a steady state level of Ca2+ release, as illustrated in Figure 3B. Other studies with rat insulinoma cells using different rates of IP3 infusion have therefore, that a combination of IP3-induced Ca2+ release and Ca2+-ATPase-mediated Ca2+ uptake causes a cycling of Ca2+ across the membrane of the endoplasmic reticulum, so that there is a regulation of the net amount of Ca2+ released by the IP3 concentration. It remains to be determined whether the activity of IP3 phosphatase is under hormonal regulation; for instance, inhibition of its activity by a GTP-binding protein or by phosphorylation would cause an increase of IP3 levels in the absence of any direct stimulation of phospholipase C activity.

The location of the subcellular structures containing the IP3-sensitive calcium pool within the cell has not been ascertained. In liver and several other, but not all, cell types the maximal amount of Ca2+ released by IP3 is considerably less than the total calcium content of the endoplasmic reticulum calcium pool. It remains possible that the IP3-sensitive Ca2+ 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 IP3-releasable Ca2+ may be distributed heterogeneously within the cell rather than in a single homogeneous calcium pool. Recent studies have used ionophoresis to introduce IP3 into frog (Xenopus laevis) eggs and have recorded the elicited Ca2+ response by Ca2+-selective microelectrodes. These studies show that although both shallow and deep injections of low concentrations of IP3 trigger local increases of intracellular free Ca2+, only shallow injections elicit a Ca2+-triggered Ca2+ release, which accompanies activation of physiological responses. This major release of Ca2+ is much greater than the IP3-induced Ca2+ release, is thought to be derived from a calcium pool adjacent to the egg cortex. The fact that IP3 injections cause small pulses of Ca2+ release independently of the site of injection suggests that the IP3-sensitive calcium pool is distributed throughout the cell and that diffusion of IP3 or Ca2+ limits the physiological response.

Mechanism of IP3-Induced Ca2+ Release

Relatively little is known about the mechanism of IP3-induced Ca2+ release. It is presumably mediated by the binding of IP3 to a specific protein in the endoplasmic reticulum membrane, but this putative IP3 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 IP3 and showed that it caused an irreversible inhibition of IP3-induced Ca2+ release in permeabilized photolabeled macrophages. Calcium release from permeabilized cells or preparations of endoplasmic reticulum is highly specific for the natural product of the PIP2 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 IP3, since glycerophosphatidylinositol-4,5-P2 and inositol-2,4,5-P3 are about 10-fold less effective than IP3 in releasing Ca2+. However, more knowledge concerning the structural requirements for IP3 analogues and potential inhibitors is urgently needed. Calcium release induced by IP3 is not impaired by vanadate inhibition of Ca2+-ATPase and does not require ATP, indicating that Ca2+ release does not occur by reversal of Ca2+-ATPase uptake. Continuous exposure to IP3 does not induce desensitization of the
Ca²⁺ release mechanism, which is also not affected by external Ca²⁺ concentrations up to 100 μM. The rate of IP₃-induced Ca²⁺ release at 37°C is on the order of 50 nmol/sec/mg of “active” endoplasmic reticulum, and the effect of lowering the temperature to 4°C is negligible. The currently known properties of IP₃-mediated Ca²⁺ release are consistent with the proposal that the binding of IP₃ to its receptor regulates a Ca²⁺ channel in specialized regions of the endoplasmic reticulum membrane and that charge compensation is achieved by an inward movement of K⁺ (S.K. Joseph and J.R. Williamson, submitted for publication, 1986).

**Kinetics of IP₃ Formation and Different Isomers**

Most measurements of the accumulation of inositol phosphates in cells after agonist stimulation have been made using cells prelabeled with [³H]inositol, and the different ³H-labeled intermediates have been separated by Dowex resin ion-exchange chromatography. With this technique, it has become apparent that different Ca²⁺-mobilizing agonists acting on the same cell stimulate phospholipase C to different degrees and that a similar maximum Ca²⁺ mobilization can be achieved with different accumulations of IP₃. Thus, although at saturating concentrations vasopressin and the α₁-adrenergic agonist phenylephrine cause a similar rapid increase of cytosolic free Ca²⁺, phenylephrine causes only a 30% increase of IP₃ 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 IP₃ being produced in the first few seconds of agonist addition to cause maximum Ca²⁺ release from the IP₃-sensitive Ca²⁺ pool, with the later accumulation of IP₃ representing overproduction.

Recent studies with parotid gland¹²⁹,¹³⁰ and hepatocytes¹³¹,¹³² have shown that two isomers of IP₃ are formed during hormonal stimulation — namely, inositol-1,3,4-P₃ and inositol-1,4,5-P₃. Measurements of the kinetics of changes in the two isomers after stimulation of cells with Ca²⁺-mobilizing agonists showed that inositol-1,3,4-P₃ was produced after the onset of inositol-1,4,5-P₃ formation and continued to accumulate for several minutes, whereas inositol-1,4,5-P₃ 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-P₃ isomer. The two observations — that it does not start to accumulate until after the peak of the intracellular Ca²⁺ transient and that the small elevation in the steady state level of the cytosolic free Ca²⁺ falls abruptly upon addition of hormone antagonist, whereas total IP₃ levels fall relatively slowly — suggest that the inositol-1,3,4-P₃ isomer is not active in Ca²⁺ mobilization. In pancreatic acinar cells and hepatocytes, high concentrations of Li⁺ (20 mM) were found to cause an increase in the agonist-stimulated accumulation of total IP₃, which was paradoxical, since the inositol-1,4,5-P₃ phosphatase was not affected by Li⁺.⁸¹,⁸² Measurements of the IP₃ isomers in pancreatic acinar cells and hepatocytes have subsequently shown that with Li⁺ pretreatment, only the inositol-1,3,4-P₃ isomer shows a further accumulation.¹³¹,¹³² indicating that only the IP₃ phosphatase specific for this isomer is Li⁺-sensitive. Very recently, higher phosphorylated forms of inositol have been detected in stimulated cells.¹³¹,¹³² In carbachol-stimulated brain slices inositol-1,3,4,5-tetrakisphosphate (IP₄) is produced as rapidly as inositol-1,4,5-P₃, and before inositol-1,3,4,5-P₄, whereas in GH₄ pituitary cells stimulated with thyrotropin-releasing hormone IP₄ does not accumulate until after inositol-1,4,5-P₃ has reached a peak (5 seconds).¹³⁴ Of the two possibilities — that IP₃ is formed by hydrolysis of phosphatidylinositol-3,4,5-P₃, or by a soluble inositol-1,4,5-P₃ kinase — the latter is more likely, since animal cells (as well as avian erythrocytes¹³⁵) contain inositol-1,4,5-P₃ kinase activity.¹³⁶ In any case, IP₃ seems to be the precursor for the inositol-1,3,4,-P₃ 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 (IP₁) and inositol-1,2-(cyclic) phosphate, with the latter compound being converted to IP₃ by a tissue hydrolase before further hydrolysis to inositol.¹³⁷ Interest in the cyclic inositol phosphates waned after it was shown that inositol-1,2-(cyclic) phosphate was unable to elicit Ca²⁺ mobilization.¹³⁸ However, it has recently been shown¹³⁹ that hydrolysis of PIP and PIP₂ 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-P₃ released Ca²⁺ 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 IP₃ in some cells.¹³⁹

**The Cytosolic Free Ca²⁺ Transient**

The typical agonist-induced effect on cytosolic free Ca²⁺ 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 Ca²⁺ indicator Quin 2.¹⁴⁰ The initial increase of Ca²⁺ is entirely due to mobilization of Ca²⁺ from the IP₃-sensitive Ca²⁺ pool, since it is unaffected by the removal of extracellular Ca²⁺ by EGTA immediately before the addition of hormone.¹⁰⁵,¹⁴¹,¹⁴² The fall of cytosolic free Ca²⁺ from its peak value coincides with a net efflux from the cell, which can occur against the physiological gradient of Ca²⁺ across the plasma membrane of approximately 10⁵. The net loss of Ca²⁺ 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 Ca²⁺ is associated with an increased uptake into cellular organelles, notably the mitochondria, where it activates pyruvate and α-ke-
phosphorylase a in hepatocytes incubated in the presence of 13

details. VP = vasopressin.

...extracellular fluid.

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...tion of phosphorylase levels. The slightly elevated a activity rapidly causes the cytosolic free Ca2+ to return rapidly to control values after the addition of hormone, in the absence of extracellular Ca2+.142

...In liver, a number of hormones, such as vasopressin, angiotensin II, and phenylephrine, when added at saturating concentrations, result in similar peak increases of cytosolic free Ca2+.141 A subsequent addition of the same or a different agonist fails to elicit any further effect. It appears, therefore, that the first addition of hormone maintains the IP3 concentration at a sufficient level to cause release of all the calcium from the IP3-sensitive Ca2+ pool for as long as a sufficient number of receptors are occupied.142 Addition of hormone antagonist at any time after the initial hormone addition causes the cytosolic free Ca2+ to return rapidly to the resting value.141,142 When another Ca2+-mobilizing agonist is added shortly after the antagonist to the first hormone, the increase in cytosolic free Ca2+ is submaximal and does not become normal until the interval between addition of the antagonist and the second hormone is extended to 5 to 10 minutes.105 This response to a second hormone addition is entirely dependent on the presence of extracellular Ca2+. Thus, once Ca2+ is released from the IP3-sensitive Ca2+ pool, it must be replenished by a net entry of Ca2+ from the extracellular fluid.

...The transient Ca2+ response illustrated in Figure 4 appears, in principle, to be typical of those observed in many cells after addition of Ca2+-mobilizing agonists, with the first (peak) phase being essentially independent of extracellular Ca2+ and the second (maintenance) phase being Ca2+-dependent. It may be noted that in instances in which the peak of the Ca2+ response is depressed in low-Ca2+ medium, relative to the control, this is probably caused by net Ca2+ efflux from the cell and loss of Ca2+ from the IP3-sensitive Ca2+ pool before addition of the agonist.3,144 The inability of agonists to cause an increase of cytosolic free Ca2+ in calcium-depleted cells is not due to an uncoupling of the receptor from phospholipase C, as shown recently with PC12 cells.144 After the cells had been treated with ionomycin and incubated in Ca2+-free medium, the addition of carbachol caused an increase of IP3, but no increase of cytosolic free Ca2+, as monitored with Quin 2. With some cells and agonists (e.g., stimulation of pituitary GH, cells by thyrotropin-releasing hormone) there is a secondary extracellular Ca2+-dependent slow rise of cytosolic free Ca2+ after the initial peak, rather than a steady state plateau value, because of a net entry of Ca2+ by a voltage-dependent Ca2+ channel.146-150 Cyclic IP3 or IP2 mediation of an agonist-induced effect on plasma membrane Ca2+ flux is an intriguing possibility that remains to be explored.131 In recent experiments with perfused rat livers where net Ca2+ fluxes across the plasma membrane induced by Ca2+ mobilizing hormones were measured with a Ca2+ electrode placed in the effluent fluid, vasopressin and angiotensin II stimulated net Ca2+ uptake after an initial net Ca2+ efflux phase, whereas phenylephrine caused only a net Ca2+ efflux while the receptors were still occupied by the hormones.156 It is possible that these differences may be related to quantitative differences between the agonist-induced accumulations of inositol lipid metabolites (e.g., of IP3 or diacylglycerol).

**Plasma Membrane Ca2+ Fluxes**

As noted in the previous section, the falling phase of the agonist-induced response of cytosolic free Ca2+ is caused by a net efflux of Ca2+ from the cell. Transport of Ca2+ out of the cell probably occurs through plasma membrane Ca2+-ATPase in response to the increase in cytosolic free Ca2+. In several different cell types (e.g., liver103 and neutrophils152) the onset of this net Ca2+ efflux is delayed relative to the rise of cytosolic free Ca2+. The efflux is caused by an inhibition of Ca2+-ATPase, which persists after isolation of plasma membrane from hormone-treated tissue.133 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 Ca2+-ATPase reconstituted into phosphatidylycholine liposomes.155 A relative inhibition of Ca2+ efflux from the cell may serve an important function in allowing the cells to retain the Ca2+ released from the IP3-sensitive Ca2+ pool during the early phase of agonist stimulation, so that the Ca2+-mediated functional effects (e.g., activation of protein kinases) can be fully expressed. Thus, once Ca2+-mobilizing hormones have been added, phosphorylase a activity in liver remains elevated long after the decline of cytosolic free Ca2+ from its peak value (see Figure 4).

Both in the resting cell and in the stimulated cell in the steady state with respect to Ca2+, there is an exchange of Ca2+ across the plasma membrane. Cal-
cium-mobilizing agonists are known to increase this plasma membrane Ca\(^{2+}\) permeability.\(^{7-9}\) 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\(_4\) 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 electrogenic 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.\(^ {163}\) Although 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 phosphatidyserine 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.\(^ {164,165}\) 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,\(^ {166,167}\) insulin secretion from pancreatic islet cells,\(^ {170,171}\) aldosterone secretion from adrenal cells,\(^ {172}\) prolactin secretion from pituitary cells,\(^ {173,174}\) amylase secretion from pancreatic acinar cells,\(^ {175}\) 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-
teins 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 secretion 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.\(^ \text{[7, 14, 154, 179]} \)

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 \(a\) levels and cytosolic free Ca\(^{2+}\).\(^ {\text{[180, 181]}} \) However, PMA has also been shown to inhibit \(\alpha_1\)-adrenergic-induced and, to a lesser extent, vasopressin-induced Ca\(^{2+}\) mobilization responses in liver.\(^ {\text{[160, 181]}} \) Chemotactic factor-induced or concanavalin A-induced Ca\(^{2+}\) mobilization in neutrophils,\(^ {\text{[182, 183]}} \) and muscarinic cholinergic effects in transformed smooth muscle (PC12) cells,\(^ {\text{[184]}} \) as well as agonist-induced formation of inositol phosphates or Ca\(^{2+}\) mobilization in platelets,\(^ {\text{[119, 185, 186]}} \) hippocampal slices,\(^ {\text{[187]}} \) astrocytoma cells,\(^ {\text{[188]}} \) and pituitary GH\(_3\) cells.\(^ {\text{[189]}} \) 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.\(^ {\text{[190]}} \) Although PMA had inhibitory effects on angiotensin II stimulation of IP\(_3\) formation and Ca\(^{2+}\) mobilization in cultured thoracic aorta smooth muscle cells,\(^ {\text{[191]}} \) 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 tissues subjected to normal agonists than in those in which the enzyme is artificially activated by PMA,\(^ {\text{[192]}} \) and this distinction may also influence the number of protein kinase C target proteins that become phosphorylated.\(^ {\text{[R.H. Cooper, R. Johanson, and J.R. Williamson, submitted for publication, 1986]}} \) In studies with hepatocytes no evidence was obtained that PMA affected cytosolic free Ca\(^{2+}\), Ca\(^{2+}\) influx, or Ca\(^{2+}\) efflux.\(^ {\text{[180]}} \) However, studies with neutrophils\(^ {\text{[182]}} \) and GH\(_3\) cells\(^ {\text{[189]}} \) 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 somatotedin, insulin, epidermal growth factor, transferrin, and histone H\(_1\), have been shown to be endogenous substrates for the enzymes.\(^ {\text{[167]}} \) 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 \(\alpha_1\)-adrenergic agonists to stimulate PI turnover, in association with a marked increase in the phosphorylation of the \(\alpha_1\)-receptor.\(^ {\text{[192]}} \) 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.\(^ {\text{[193]}} \)

In contrast, the apparent effect of protein kinase C in increasing PI and PIP\(_2\) kinase activation may provide positive feedback for agonist stimulation, since these enzymes are primarily responsible for maintaining the phosphoinositide levels as precursor substrates for generation of IP\(_3\) and diacylglycerol. So far, the phenomenon has been reported only in platelets\(^ {\text{[186, 184, 195]}} \) and thyocytes,\(^ {\text{[196]}} \) 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.\(^ {\text{[193]}} \)

Various growth factors, which were previously shown to stimulate inositol lipid metabolism,\(^ {\text{[197]}} \) cause an elevation of cytosolic free Ca\(^{2+}\),\(^ {\text{[198]}} \) which is thought to be a requirement for cell proliferation.\(^ {\text{[11, 192]}} \) 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,\(^ {\text{[199-205]}} \) 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.\(^ {\text{[206]}} \) 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.\(^ {\text{[207]}} \) 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-
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⁺ permeabilization, 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 with 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.

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