Turnover of Inositol Phospholipids and Signal Transduction

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Summary. Various extracellular informational signals such as those from a group of hormones and some neurotransmitters appear to be passed from the cell surface into the cell interior by two routes, protein kinase C activation and Ca^{2+} mobilization. Both routes usually become available as the result of an interaction of a single ligand and a receptor and act synergistically to evoke subsequent cellular responses such as release reactions. The signal-dependent breakdown of inositol phospholipids, particularly phosphatidylinositol bisphosphate, now appears to be a key event for initiating these processes.

In the late 1940's, polyphosphoinositides were isolated from brain by Folch (*1*). Subsequently they were shown to consist of diphosphoinositide (phosphatidylinositol 4-phosphate, PIP) and triphosphoinositide (phosphatidylinositol 4,5-bisphosphate, PIP_2) (*2*). Both PIP and PIP_2 (each represents less than a few percent of the total inositol phospholipids) are produced from phosphatidylinositol (PI) in situ through sequential phosphorylation of its inositol moiety by the actions of PI and PIP kinases (*3*). In brain tissues polyphosphoinositides appear to be localized to two distinct pools, a metabolically stable pool associated with myelin and a labile pool in neuronal, and perhaps also glial, plasma membranes (*4*). The phosphoryl groups covalently attached to these phospholipids turn over extremely rapidly, but their physiological roles in signal transduction have not been elucidated until recently.

A wide variety of extracellular signals (*5*), such as those exemplified in Table 1, induce inositol phospholipid turnover in their respective target tissues. Initially, PI was regarded as a prime target (*6*), but recent evidence seems to suggest that, after stimulation of the receptor, PIP_2 rather than PI and PIP is degraded immediately to produce 1,2-diacylglycerol and *demyo*-inositol 1,4,5-trisphosphate (IP_3) (*7*) (Fig. 1). In general, this turnover of membrane phospholipids is associated with an increase in intracellular concentration of Ca^{2+}, which appears to mediate many of the subsequent physiological responses. Studies in this laboratory have provided evidence that the diacylglycerol produced in this way initiates the activation of a specialized protein kinase that we call protein kinase C, and that the information of extracellular signals may be directly transferred across the membrane to result in protein phosphorylation.

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In this article the evidence will be briefly summarized for the activation of protein kinase C, and for the importance of that activation for the subsequent cellular responses. The activation of cellular functions by this route appears to be separate from and synergistic to those activated via an increase in intracellular Ca^{2+}. Although the best evidence supporting this view has primarily come from a series of experiments with platelets, the results outlined in this article may provide some indication of the physiological roles of the turnover of inositol phospholipids in signal transduction in neuronal tissues. Several aspects relating to this protein kinase system have been reviewed (*8*).

Link to Protein Phosphorylation

Protein kinase C is widely distributed in tissues and organs, with the brain having the highest specific activity (*9*). In brain, a large quantity of the enzyme is associated with synaptic membranes, whereas in most other tissues the enzyme is recovered mainly in the soluble fraction as an inactive form when fractionated into subcellular components (*10*). When assayed in a cell-free enzymatic reaction system, the protein kinase depends on Ca^{2+} as well as phospholipid, particularly phosphatidylserine, for its activation. However, diacylglycerol, which is produced in membranes from inositol phospholipids in a signal-dependent fashion, increases the affinity of this enzyme to Ca^{2+}, so that as little as $10^{-7}M$ is required, and thereby renders it fully active without a net increase in the Ca^{2+} concentration (*11*). As will become evident later, the activation of protein kinase C is thus biochemically dependent on, but physiologically independent of, the divalent cation. In physiological processes, the enzyme appears to be activated by interaction with a locus in the membrane phospholipid bilayer that is specifically produced by diacylglycerol.

When platelets are stimulated by thrombin, collagen, or platelet-activating factor (PAF), various constituents of platelet granules are released, such as serotonin and adenine nucleotides of dense bodies, β-thromboglobulin and platelet-derived growth factor (PDGF) of α-granules, and acid hydrolases of lysosomes. These release reactions are associated with phosphorylation of predominantly two endogenous platelet proteins with approximate molecular weights of 40,000 and 20,000 (40K and 20K) (*12, 13*). The 20K protein appears to be identical to the myosin light chain, and the enzyme responsible for this reaction is calmodulin-dependent and absolutely requires mobilization of Ca^{2+} (*14, 15*). On the other hand, protein kinase C is identified as the enzyme that is responsible for the phosphorylation of the 40K protein (*13*). This protein has been highly purified and shown to be a 47K component which displays some microheterogeneity as judged from isoelectrofocusing data (*16*). The function of the 47K protein in release reactions remains unknown.

When stimulated, platelets rapidly produce a diacylglycerol, which comprises (mostly) the 1-stearoyl-2-arachidonyl backbone (*17*), and this reaction is accompanied by the concomitant disappearance of inositol phospholipids (*13, 18*). This diacylglycerol is present in membranes only transiently; within a minute of formation it disappears, either returning to inositol phospholipids or becoming further degraded to arachidonic acid for thromboxane and prostaglandin synthesis. This transient appearance of diacylglycerol in membranes is always

associated with protein kinase C activation as judged by 40K protein phosphorylation (*13*).

In the in vitro enzymatic reaction, various synthetic diacylglycerols are equally capable of activating the enzyme. The diacylglycerols having two long fatty acyl moieties such as diolein cannot be readily intercalated into cell membranes. When one of the fatty acids is replaced by an acetyl group, the resulting diacylglycerol, 1-oleoyl-2-acetylglycerol, which has some detergent-like properties, can be dispersed easily into the phospholipid bilayer and could thus activate protein kinase C in intact cells directly (*19*). Under these conditions, neither the breakdown of inositol phospholipids, the formation of the endogenous diacylglycerol, nor the release of arachidonic acid is induced. There is no sign of damage to cell membranes. This diacylglycerol is metabolized very rapidly in situ to its corresponding phosphatidic acid, 1-oleoyl-2-acetyl-3-phosphorylglycerol. Platelets are particularly suited for demonstrating such direct activation of protein kinase C, since the phosphorylation of a specific endogenous substrate, 40K protein, may be used as an indicator. Two-dimensional electrophoretic (fingerprint) analysis of the 40K protein preparations phosphorylated in vivo and in vitro confirms that protein kinase C is indeed activated. No Ca^{2+} is mobilized as measured indirectly by the phosphorylation of 20K protein (*19*), or directly as measured by an intracellular Ca^{2+} indicator, quin 2 (*20*). On the basis of these observations, together with the evidence obtained with the activators and inhibitors described below, it may be concluded that diacylglycerol transiently produced from inositol phospholipids serves as the direct activator of protein kinase C. The signal-induced transient accumulation of diacylglycerol has also been observed for other tissues, including pancreas stimulated by acetylcholine (*21*), mast cells stimulated by polycationic compound 48/80 (*22*), thyroid follicles stimulated by thyrotropin (*23*), Swiss mouse 3T3 cells stimulated by PDGF (*24*), aortic endothelial cells stimulated by bradykinin (*25*), pituitary cells stimulated by thyrotropin-releasing hormone (*26*), and hepatocytes stimulated by vasopressin (*27*).

Table 1. Messengers that induce inositol phospholipid turnover. In addition to those listed, a large number of substances and stimulants that induce activation of cellular functions and proliferation are known to induce inositol phospholipid turnover (*5*).

Acetylcholine (M1)
Norepinephrine (α1)
Epinephrine (α1)
Dopamine (D2?)
Histamine (H1)
Serotonin (5HT2?)
ACTH (?)
Vasopressin (V1)
Angiotensin II
Cholecystokinin
Cerulein
Gastrin
Bombesin
Pancreozymin
Substance P
Bradykinin
Thromboxane
Thrombin
Collagen
Platelet-activating factor
K^+ depolarization
Electrical stimulation
Chemoattractants
Secretagogues
Growth factors
Mitogens

Activators and Inhibitors

Tumor-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate mimic the action of hormones and neurotransmitters in various tissues, and several kinetic studies suggest that the primary site of their actions is the cell surface membrane (*28*). Studies in this laboratory have shown that protein kinase C is a target for phorbol esters, because the tumor promoters act as a substitute for diacylglycerol and directly activate this protein kinase both in vitro and in vivo (*29*). The structural requirements of phorbol-related diterpenes for tumor promotion are roughly similar to those for the activation of protein kinase C. Our subsequent studies and those of others seem to indicate that some, if not all, of the pleiotropic actions of tumor-promoting phorbol esters are mediated through the action of protein kinase C (*30, 31*). Thus, the tumor promoters appear to be used as specific activators of this enzyme at cellular and subcellular levels. The brain is a rich source of phorbol ester receptor, and the amount of this receptor roughly matches the amount of protein kinase C (*30*).

A kinetic study with homogeneous protein kinase C, obtained from rat brain (*10*), suggests that one molecule of the tumor promoter, which enters the membrane phospholipid bilayer by intercalation, activates one molecule of the enzyme (*30*). This raises the possibility that, in physiological processes, one molecule of diacylglycerol produced from inositol phospholipids in membranes activates one molecule of protein kinase C. This stoichiometry does not necessarily indicate that the tumor promoter or diacylglycerol activates the enzyme by a direct ligand-ligand interaction. Rather, it may be that each molecule of the phorbol ester or diacylglycerol modifies a phospholipid microenvironment with which the protein kinase C produces a quaternary complex composed of the enzyme, phospholipid, Ca^{2+}, and tumor promoter or diacylglycerol, which is fully active for protein phosphorylation (*30*). The precise nature of this hydrophobic interaction remains unknown. However, some tyrosine-specific protein kinases encoded by the oncogenes *src* (*32*) and *ros* (*33*) have been proposed to enhance the phosphorylation of PI and PIP, thereby increasing the availability of PIP_2 in cell surface membranes.

There appears to be no inhibitor that is specific for protein kinase C, although several chemicals and materials inhibit the enzyme action (*34*). For example, the enzyme can be inhibited to various extents by psychotropic drugs (such as trifluoperazine, chlorpromazine, fluphenazine, and imipramine), local anesthetics (dibucaine and tetracaine), W-7, verapamil, phentolamine, adriamycin, polyamines (spermine, spermidine, and putrescine), palmitoylcarnitine, melittin, heparin, polymixin B, vitamin E, and retinoic acid. The inhibition of protein kinase C by these drugs is not due to their interaction with the active center of the enzyme since catalytically active enzyme fragment obtained by limited proteolysis (*35*) is not susceptible to any of these drugs. Kinetically, most of the drugs listed above interact with phospholipid and thereby inhibit the activation of enzyme in a competitive manner (*36*).

The phospholipid-interacting drugs usually inhibit calmodulin-dependent protein kinases such as myosin light chain kinase by competing with calmodulin. In an experiment with platelets, some of these drugs such as chlorpromazine, dibucaine, and tetracaine did not inhibit thrombin-induced diacylglycerol formation, but profoundly inhibited the activation of protein kinase C in a dose-dependent manner (*13*). Cyclic nucleotide–dependent protein kinases are not susceptible to these drugs.

Mobilization of Calcium

Except possibly for tissues such as bovine adrenal medullary cells (*37*) and for presynaptic muscarinic receptors (*38*), it is generally the case that, when stimulation of receptors leads to inositol

phospholipid breakdown, Ca^{2+} is mobilized simultaneously. Several groups of investigators have obtained some evidence that IP_3 could serve as a mediator for Ca^{2+} release from intracellular, most likely nonmitochondrial stores. For example, using pancreatic acinar cells which are permeabilized to highly charged molecules such as IP_3, Streb *et al.* (*39*), using a Ca^{2+}-specific electrode assay, showed that this compound releases Ca^{2+} from the same stores as acetylcholine does. Similar results have been obtained for saponin-permeabilized hepatocytes (*40*) and also for skinned single cells of coronary artery (*41*) as assayed with a Ca^{2+}-specific electrode or more directly with radioactive Ca^{2+}. Thus, it is possible that IP_3, one of the products of signal-induced breakdown of PIP_2, may increase Ca^{2+} levels within the cells. If this proposal is correct, then one cycle of inositol phospholipid turnover may generate two intracellular mediators, diacylglycerol and IP_3, for subsequent signal transduction with the expenditure of four molecules of adenosine triphosphate (ATP) (Fig. 1).

The phospholipases C in mammalian tissues, except for the enzyme of lysosomal origin, normally require high concentrations of Ca^{2+} when assayed in vitro. Although the regulatory mechanism of this enzyme is uncertain, various results so far reported are compatible with the supposition that inositol phospholipid turnover is Ca^{2+}-dependent but may not be regulated by Ca^{2+}. A typical example for such Ca^{2+} sensitivity modulation of enzyme may be referred to protein kinase C, whose activity is dependent on Ca^{2+} but is regulated by diacylglycerol as described above. Perhaps, there might be more than one mechanism eventually leading to the required intracellular concentration of Ca^{2+} for full activation of cellular functions, particularly for long-term cellular responses.

Synergistic Role of Two Routes

The activation of protein kinase C appears to be a necessary but not a sufficient requirement for physiological responses of target cells, because the cellular responses to synthetic diacylglycerol or to tumor promoter per se are always incomplete. Under appropriate conditions it is possible to induce protein kinase C activation and Ca^{2+} mobilization selectively by the exogenous addition of diacylglycerol or tumor promoter for the former and a Ca^{2+}-ionophore, such as A23187, for the latter. When platelets are used for this demonstration, these two routes can be specifically monitored by measuring the phosphorylation of the two endogenous proteins, the 40K and 20K proteins, as mentioned earlier. By contrast, when platelets are stimulated by extracellular signals such as thrombin, both proteins are phosphorylated at the same time. Thus these procedures can be used to show that protein kinase C activation and Ca^{2+} mobilization are both essential and that they act synergistically to elicit full physiological responses such as release reactions (*19*). In these experiments the concentration of A23187 (0.2 to 0.4 μM) is critical,

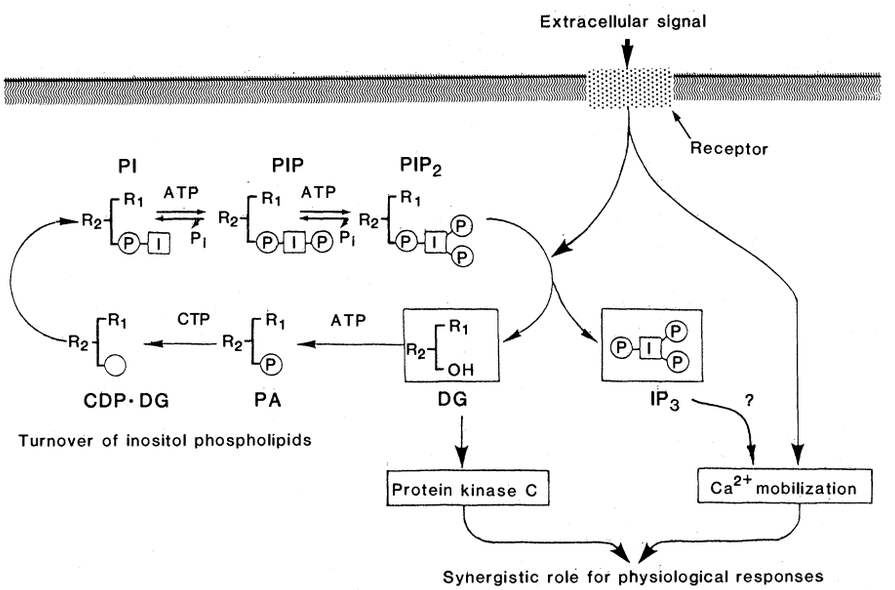

Fig. 1. Turnover of inositol phospholipids and signal transduction. Inositol phospholipids appear to be in equilibrium in membranes, although polyphosphoinositides are normally very minor components. Recent evidence obtained for several tissues, such as iris smooth muscle, hepatocytes, parotid gland, brain, adrenal gland, platelets, and blowfly salivary gland, indicates that phosphatidylinositol 4,5-bisphosphate is the immediate target of the signal-dependent hydrolysis (*7*). The enzymological basis and tissue variation of this reaction remain to be determined. PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DG, 1,2-diacylglycerol; IP_3, *demyo*-inositol 1,4,5-trisphosphate; I, inositol; P, phosphoryl group; and R_1 and R_2, fatty acyl groups.

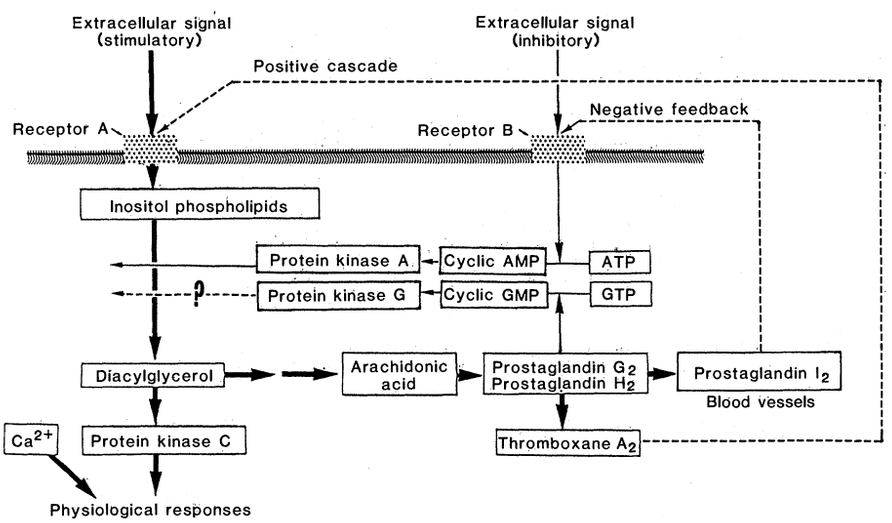

Fig. 2. Possible cascade and feedback control of cellular functions. Platelets are used as a model system. Thromboxane A2 produced in a stimulated platelet will cause a cascade of activation of other platelets by interaction with their cell surface receptor, which provokes inositol phospholipid breakdown. By contrast, prostaglandin I_2 produced in blood vessels will prevent further activation of platelets by elevating cyclic AMP. The exact signal pathway to increase cyclic GMP and the role of this cyclic nucleotide have not yet been unequivocally established. Protein kinase A, cyclic AMP–dependent protein kinase; protein kinase G, cyclic GMP–dependent protein kinase.

Table 2. Physiological responses possibly caused by synergistic action of protein kinase C activation and Ca^{2+} mobilization.

Tissue	Physiological response	References
Platelets	Serotonin release	(*19, 68*)
	Lysosomal enzyme release	(*68, 69*)
Neutrophils	Lysosomal enzyme release	(*69*)
Adrenal medulla	Epinephrine secretion	(*70*)
Adrenal glomerulosa	Aldosterone secretion	(*71*)
Pancreatic islets	Insulin secretion	(*72*)
Pancreatic acinar cells	Amylase secretion	(*73*)
Mast cells	Histamine release	(*74*)
Ileal cholinergic nerve	Acetylcholine release	(*45*)
Hepatocytes	Glycogenolysis	(*46*)
T lymphocytes	DNA synthesis	(*47*)

because at higher concentrations (more than 0.5 μM) this Ca^{2+} ionophore itself will cause the phosphorylation of 40K protein in addition to 20K protein, because of the nonspecific activation of phospholipase C as well as of protein kinase C by a large increase in Ca^{2+} concentration. Likewise, the synthetic diacylglycerol or tumor promoter alone at higher concentrations (50 μg/ml or 50 ng/ml, respectively) causes a significant release of platelet constituents without a measurable increase in the Ca^{2+} concentration. The precise reason for this enhanced release is unclear, but it is possible that these compounds can induce the release reaction by acting as membrane fusigens or perturbers or by generating superoxide (or both).

The involvement of the two synergistic routes in the activation of cellular functions may explain, at least in part, the signal selectivity that is often observed for release reactions. In platelets, for example, serotonin and adenine nucleotides are released from dense bodies in response to such signals as thrombin, collagen, adenosine diphosphate (ADP), epinephrine, and PAF, while lysosomal enzymes are released only at higher concentrations of thrombin and collagen. By using permeabilized platelets Knight *et al.* (*42*) have shown that such signal selectivity of release reactions is not related to Ca^{2+} concentrations, because there is no difference in their sensitivity to Ca^{2+}. It is theoretically possible that the two routes mentioned above may exert differential control over release reactions from different granules within a single activated platelet. In neuronal tissues, a single nerve ending frequently contains both peptides and classical transmitters presumably in different stores (*43*). It is possible that the two synergistic routes may also be responsible for the frequency selectivity of release reactions that is often observed during electrical stimulation. Depolarization of membranes by electrical stimulation or depolarizing agents is well known to induce inositol phospholipid turnover (*44*).

The synergistic role of protein kinase C and Ca^{2+} mobilization does not appear to be confined to the platelet, but has been suggested for several other systems (Table 2). In guinea pig ileum, the combination of A23187 and tumor-promoting phorbol ester induces full activation of acetylcholine release from the cholinergic nerve endings (*45*). In this experiment the tumor promoter is shown to potentiate the ionophore-induced response approximately threefold, while the promoter alone shows no obvious effect on this release reaction. The physiological responses may include not only release reactions of various constituents of many cell types but also hepatic glycogenolysis (*46*) and lymphocyte growth responses (*47*).

Target Proteins in Brain Tissue

Although evidence is accumulating to suggest the potential importance of protein kinase C, little is known about its physiological target proteins in most tissues. In in vitro systems, the enzyme has broad substrate specificity, and phosphorylates seryl and threonyl residues, but not tyrosyl residues, of many endogenous proteins. In neuronal tissues, B50 protein (F1 protein), which is associated specifically with presynaptic membranes (*48*), serves as a preferable substrate specific to protein kinase C (*49*). This protein phosphorylation selectively increases after long-term potentiation in the hippocampal neural activity, and may be related to the expression of synaptic plasticity (*50*). Synapsin I, another protein located specifically on the cytoplasmic surface of synaptic vesicles, serves as a substrate for cyclic adenosine monophosphate (cyclic AMP)–dependent protein kinase (protein kinase A) as well as for calmodulin-dependent protein kinases (*51*). Protein kinase C also reacts with this protein as a phosphate acceptor (*52*), suggesting that it is one of the possible targets of protein kinase C, and plays some roles in release reactions. Microtubule-associated proteins (*53*), 87K protein present in synaptosomal cytosol (*54*), myelin basic protein (*53, 55*), many unidentified soluble and membrane-bound proteins in nervous tissues (*56*) are shown to be phosphorylated by protein kinase C, but the physiological significance of these reactions is unclear.

Protein kinase C apparently lacks tissue specificity. Protein kinases C and A often share many phosphate acceptor proteins in vitro, but with very different relative rates of phosphorylation of different aminoacyl residues. Obviously in many tissues, these protein kinases display distinctly different functions, but their precise targets in nervous tissues are still to be explored further.

Relationship to Other Receptors

In receptor mechanisms there may be extensive heterogeneity and many variations from tissue to tissue, but most tissues seem to have at least two major classes of receptors for transducing information across the membrane. One class is related to cyclic AMP, while the other provokes rapid turnover of inositol phospholipids as well as mobilization of Ca^{2+}. In addition, the stimulation of the latter class of receptors normally leads to arachidonate and its metabolites, and often increases cyclic guanosine monophosphate (GMP). Thus, protein kinase C activation, Ca^{2+} mobilization, arachidonic acid release, and cyclic GMP formation appear to be integrated in a single receptor cascade (*5, 8, 57*).

The mode of cellular responses may be roughly divided into two groups. In bidirectional control systems in many tissues such as platelets, the signal that induces the turnover of inositol phospholipids promotes the activation of cellular functions, whereas the signal that produces cyclic AMP usually antagonizes such activation (Fig. 2). In platelets, the signal-induced inositol phospholipid breakdown, diacylglycerol formation, 40K protein phosphorylation, and serotonin release are all blocked concurrently and progressively by increasing amounts of prostaglandin E1 as well as dibutyryl cyclic AMP (*13, 53*). Similar inhibitory actions of cyclic AMP have been demonstrated in many other bidirectional control systems. This inhibitory action of cyclic AMP may extend to the mobilization of Ca^{2+}, presumably

through activation of protein kinase A (*58*). However, the molecular basis of this counteraction in or near the membrane remains to be explored.

In contrast, in monodirectional control systems in some tissues, such as hepatocytes and perhaps certain endocrine cells, the two classes of receptors appear to function independently. In hepatocytes, inositol phospholipid turnover that is induced by α-adrenergic stimulators is not blocked by β-adrenergic stimulators nor by dibutyryl cyclic AMP (*59*). Both α- and β-stimulators cause glycogenolysis in the liver. In some other tissues such as pineal gland, the cellular responses to β-adrenergic stimulators are potentiated by α-adrenergic stimulators that induce inositol phospholipid turnover and Ca^{2+} mobilization (*60*). Presumably, in such tissues protein kinase C potentiates the adenylate cyclase system or acts cooperatively with protein kinase A to induce full cellular responses.

Arachidonic acid is derived from inositol phospholipids through two consecutive reactions catalyzed initially by phospholipase C and subsequently by diacylglycerol lipase (*61*), since inositol phospholipids in mammalian tissues contain mostly arachidonic acid at the position 2 (*17*). This fatty acid may also be released from phosphatidylethanolamine as well as from phosphatidylcholine. Perhaps, when the receptor is stimulated, both phospholipases C and A_2 act in concert, but the physiological picture of this receptor-linked release of arachidonic acid is not fully understood.

Although Ca^{2+} causes direct activation of guanylate cyclase in some tissues (*62*), it seems likely that arachidonic acid peroxide and prostaglandin endoperoxide serve as activators for this enzyme (*63*). A function of cyclic GMP may be to act as a "negative" rather than as a "positive" intracellular mediator, thus providing an immediate feedback control that prevents over-response. This proposal is based primarily on the observations that sodium nitroprusside, which induces a marked elevation of cyclic GMP, is a powerful inhibitor of platelet activation (*64*). Indeed, like cyclic AMP, the 8-bromo derivative of cyclic GMP, as well as sodium nitroprusside, can inhibit the thrombin-induced biochemical events and thereby counteract the activation of protein kinase C in platelets (*65*). Conversely, when platelets that are permeabilized for charged molecules of low molecular weight, cyclic GMP increases the sensitivity of platelets to Ca^{2+} for their release of serotonin (*66*). Although some functions of cyclic GMP and cyclic GMP–dependent protein kinase have been suggested for nervous tissues (*67*), crucial information on the role of this cyclic nucledotide is still lacking.

Implication and Conclusion

Although experimental support for the potential role of the turnover of inositol phospholipids in signal transduction has resulted from studies with a limited number of specific tissues, primarily with platelets, the evidence outlined in this article suggests its crucial importance in nervous tissues. It is possible that, as in platelets, the two routes of information flow, protein kinase C activation and Ca^{2+} mobilization, which may be opened by a single receptor stimulation as well as by depolarization, are directly and synergistically involved in neurotransmitter release in many neuronal processes. Presumably, the receptors relating to cyclic AMP regulate these processes positively or negatively in the manner briefly outlined above. A major role of protein kinase C in the release reactions is probably to modulate their sensitivity to Ca^{2+}.

It is obviously possible that the role of protein kinase C may also be extended to the modulation of membrane conductance, channels and active transport, signal-receptor interaction, axoplasmic flow, neurotransmitter biosynthesis, and other neuronal functions by phosphorylating the proteins involved. One might anticipate that the signal-induced turnover of inositol phospholipids that is initiated in a single cell surface membrane is directly related not only to the control of its own cellular functions, but also to the regulation of other homologous and heterologous cell types through the action of various metabolites of the phospholipids such as diacylglycerol, arachidonic acid, and prostaglandins.

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