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NEUROTRANSMITTER RECEPTORS AND PHOSPHOINOSITIDE TURNOVER*

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INTRODUCTION

Neurotransmitters, neuromodulators, hormones and growth-promoting factors are now known to exhibit their diverse metabolic and physiological responses by interaction with their selective receptors located on the cell surface. These surface receptors transduce and amplify extracellular signals by the generation of so-called second messengers. Among these are inositol phosphates and 1,2-diacylglycerol (DG) which are formed by hydrolysis of membrane-bound phosphoinositides in response to receptor stimulation. This dual messenger system has attracted enormous attention in recent years because of its involvement in an array of physiological processes, including neuronal membrane excitability, synaptic transmission, cellular metabolism, muscle contraction, sensory transduction, motility, growth, DNA synthesis, and platelet aggregation. Several recent articles review various aspects of this receptor-signaling system (1–15). This review focuses on recent developments concerning the metabolism of second messengers, their mode of action, the regulation of neurotransmitter receptor signalling, and the interaction with other receptor-coupled effectors. The explosive growth of the field of phosphoinositide turnover has touched virtually every major biological system, and a detailed review of each system is impractical. I therefore concentrate on a few systems, in particular the CNS and related neural tissues where the metabolism of phosphoinositides is particularly active.

Phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4, 5-bisphosphate (PIP_2) are collectively referred to as

phosphoinositides in this chapter. In eukaryotic cells, phosphoinositides constitute less than 10% of total phospholipids (6–8% for PI, 1% for PIP, and 0.5% for PIP_2). However, their metabolic rates are far greater than those of other phospholipids in the cell. Most of PIP and PIP_2 and a minor fraction of PI are plasma membrane-bound, while the majority of PI is present in intracellular organelles, especially endoplasmic reticulum. PIP and PIP_2 are formed in the membrane plane via sequential phosphorylation of PI by the enzymes PI kinase and PIP kinase, respectively. Although it has been about three decades since Mabel and Lowell Hokin (16) discovered that stimulation of muscarinic acctylcholine receptors provoked a striking enhancement of the turnover of PI and its precursor phosphatidic acid, only within the last six to eight years has a coherent scheme of how this occurs bcgun to emerge. It is now generally accepted that stimulation of over 20 types of cell-surface receptors leads to hydrolysis of PIP_2 by phospholipase C (or phosphodiesterase) with the formation of inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) and DG. $Ins(1,4,5)P_3$ is released into the cytoplasm to mobilize calcium from nonmitochondrial stores (2, 17), while DG remains in the plasma membrane plane and activates protein kinase C (5, 11). This dual messenger pathway involving $Ins(1,4,5)P_3$ and DG has evolved as a mechanism designed to control a spectrum of diverse physiological events.

GENERATION OF INOSITOL PHOSPHATES AND DIACYLGLYCEROL

Hydrolysis of Phosphoinositides

Accumulating evidence indicates the presence of hormone-sensitive and insensitive pools of phosphoinositides in a variety of systems. The turnover of the hormone-sensitive pool is activated by hormones and neurotransmitters (2), while thc metabolism of the insensitive pool is slow, but can bc altered by manganese (18, 19), calcium, (19), and ionophores (20). These observations suggest that the syntheses of the two pools are compartmentalized. It is generally thought that the active pools of phosphoinositides are located in the plasma membrane where receptor agonist-mediated hydrolysis of phosphoinositides takes place. The synthetic enzyme, PI synthetase, is present in endoplasmic reticulum and the newly synthesized PI is transported to the plasma membrane by protein exchange (for review, see 21). Once it reaches the plasma membrane, PI can be sequentially phosphorylated by specific ATP-dependent kinases, first to form PIP then PIP_2. These reactions can be reversed by step-wise dephosphorylation through phosphatases acting on PIP_2 and PIP.

Compelling evidence indicates that plasma membrane-bound PIP_2 is the substrate for phospholipase C. This is supported by unequivocal demonstra-

tion of the generation of Ins(1,4,5)P_3 in numerous systems and the involvement of Ins(1,4,5)P_3 in many critical physiological events. However, there is increasing documentation suggesting that under certain conditions, PI and PIP can also be hydrolyzed, a concept reviewed by Majerus et al (8, 15). It is likely that, in most cases, hydrolysis of PI and PIP is an event triggered by Ins(1,4,5)P_3-induced mobilization of calcium due to stimulus-mediated breakdown of PIP_2. Changes in the membrane lipid microenvironment as a result of PIP_2 hydrolysis may also contribute to the change in substrate specificity from PIP_2 to PIP or PI. Since the majority of PI is located in the endoplasmic reticulum, it is also possible that some PI hydrolysis may take place in this organelle, secondary to the stimulation occurring on the plasma membrane. Since PI is 10 times more abundant than PIP_2 in most tissues, the physiological significance of direct hydrolysis of PI and PIP by phospholipase C cannot be ignored. This event would lead to the massive and sustained production of DG with the consequent persistent activation of protein kinase C and thus provide a mechanism for activating protein kinase C continuously without further mobilization of cellular calcium.

Phospholipase C

This enzyme exists mainly as a soluble form, although it has also been described and characterized as membrane-bound. Multiple forms have been reported in numerous tissues and cell types. Available evidence indicates these forms hydrolyze all types of phosphoinositides, but with different calcium requirements and substrate preferences, e.g. in bovine brain cytosol, at least three immunologically distinct forms of phosphoinositide-specific phospholipase C catalyze the hydrolysis of PIP_2, PIP, and PI (22, 23). When calcium concentration is in the micromolar range, PIP and PIP_2 are better substrates than PI for all three types of enzymes; however, in millimolar concentrations of calcium, the hydrolysis of PI is markedly accelerated (23). Similar calcium requirements for PIP_2 and PI hydrolysis were observed in platelets (24–26), rat liver and brain (27), guinea pig uterus (28), and rat aorta (29). In bovine brain, type I phospholipase C appears to be equally distributed between the cytosolic and membrane fractions, while virtually all types II and III are in the cytosolic form (22, 30). Thy hydrolysis of PIP_2 by the type I, but not type II, is markedly stimulated by GTP in low calcium (μM range) (23). Guanine nucleotides have also been shown to activate PIP_2 hydrolysis by membrane-bound phospholipase C in rat liver (31) and aorta (29), and PIP_2 hydrolysis by cytosolic phospholipase C in human platelets and calf brain (25). These results suggest the involvement of a guanine nucleotide binding protein in this activation (see below). In pineal glands, the highest specific activity of the hydrolysis of PIP_2 and PIP is associated with the membrane fraction, where activity has two pH optima at 5.5 and 7.5, and the cytosolic

fraction only one at 5.5 (32). This suggests that under physiological conditions, the primary site of the lipase C action may be within the plasma membrane. It is reasonable to assume that some cytosolic phospholipase C can be translocated to the membrane following stimulation. However, neither this phenomenon nor the factor(s) involved in translocation has been elucidated. Some of the discrepancies in the characteristics, regulation, and distribution of this enzyme may stem from differences in assay conditions and procedures used to prepare the enzymes. Immunohistochemical localization revealed that both type I and type II phospholipase C are expressed in neurons of rat brain. The type II enzyme immunoreactivity is localized in nearly all neurons in all brain areas, while the type I enzyme has a much more restricted distribution with the highest density in the striatum and their afferent projections to the globus pallidus and substantia nigra (33). Recently, Stahl et al (34) reported the cloning of a bovine brain complementary DNA encoding a phospholipase C of 148 kD that hydrolyzes PI when expressed in mammalian cells. Interestingly, a striking sequence similarity exists between specific regions of the lipase and the non-catalytic domain of non-receptor tyrosine kinases. This sequence homology between two functionally distinct effectors might suggest that they interact with a common molecular entity within the cell.

Metabolism of Inositol Phosphates

DG formed by hydrolysis of phosphoinositides remains in the hydrophobic membrane plane and is metabolically unstable. It can be rapidly phosphorylated to form phosphatidic acid for the resynthesis of PI via the CDP-DG pathway, or it can be degraded by DG lipase to liberate arachidonic acid used for the production of other messengers, i.e. prostaglandins, leukotrienes, and thromboxanes. Thus, generation of the second messenger, DG, triggers the synthesis of other messengers and may result in multiple amplifications of the initial signal received by the calcium-mobilizing receptors.

The metabolism of inositol phosphates is proving exceedingly complex. Ins(1,4,5)P_3 resulting from PIP_2 hydrolysis can be metabolized by sequential dephosphorylation by a 5-phosphatase to form first Ins(1,4)P_2 and then Ins(4)P_1 (35). Alternatively, Ins(1,4,5)P_3 can be phosphorylated to Ins(1,3,4,5)P_4 (36, 37) that is then dephosphorylated by a similar, but probably not identical, 5-phosphatase to form an isomeric inositol trisphosphate, Ins(1,3,4)P_3 (37, 38). The scheme outlined in Figure 1 illustrates major steps involved in the metabolism of various isomers of inositol phosphates in eukaryotic cells. Ins(1,4,5)P_3 3-kinase catalyzes the phosphorylation to Ins(1,3,4,5)P_4, an enzyme now known to be activated by calcium and calmodulin (39–41). Mobilization of intracellular calcium after $InsP_3$ generation may

activate the 3-kinase, and thereby facilitate the elimination of $Ins(1,4,5)P_3$ and the termination of its biological activity. $Ins(1,3,4)P_3$ can release intracellular calcium, as does $Ins(1,4,5)P_3$, but is much weaker in potency (42). $Ins(1,3,4,5)P_4$ has been shown to influence the entry of external calcium as well as to potentiate the response to $Ins(1,4,5)P_3$ (see below). Thus, the pathway involving $Ins(1,4,5)P_3$ phosphorylation may provide a mechanism by which additional intracellular second messengers are generated. $Ins(1,3,4)P_3$ has reportedly been used to produce a new tetrakisphosphate isomer $Ins(1,3,4,6)P_4$ in adrenal glomerulosa cells stimulated with angiotensin II (43). $InsP_5$ and $InsP_6$ are also present in relatively high concentrations in most mammalian cells. Vallejo et al (44) demonstrated that $Ins(1,3,4,5,6)P_5$ and $InsP_6$ are synthesized in intact brain after labeling with ^{3}H-inositol in vivo. Moreover, local infusion of these two inositol polyphosphates into a discrete brain stem nucleus results in changes in heart rate and blood pressure, suggesting that they could be novel tools for the study of central control of cardiovascular function(44).

Most pathways involved in the dephosphorylation of inositol phosphates have been discussed in recent reviews (8, 15, 45), but rapid expansion of work in this area during the last two years has clarified some new steps. In liver (46), brain (47, 48), GH_4, (49), and GH_3 (50) cells, for instance, $Ins(1,3,4)P_3$ is degraded to $Ins(3,4)P_2$ and $Ins(1,3)P_2$. A lithium-sensitive inositol polyphosphate 1-phosphatase in brain homogenates appears to hydro-

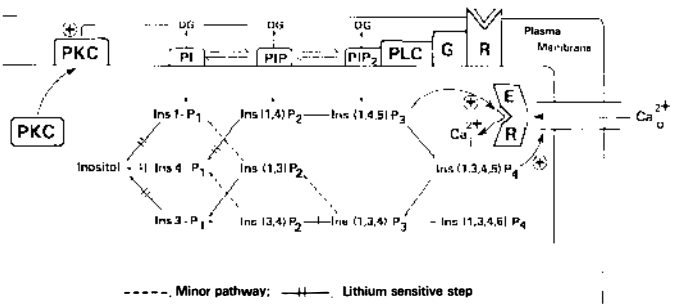

Figure 1 Schematic representation of the metabolism of inositol phosphates released from phosphoinositides. Activation of the receptor (R) coupled to phospholipase C (PLC) through a guanine nucleotide binding protein (G) leads to hydrolysis of PIP_2, forming two key metabolites, DG and $Ins(1,4,5)P_3$. Under certain conditions, hydrolysis of PIP and PI may also occur following receptor stimulation. DG stays in the plasma membrane plane to activate protein kinase C (PKC), which is translocated to the membrane after stimulation. $Ins(1,4,5)P_3$ mobilizes intracellular calcium ($Ca^{2+}{}_i$) store from the endoplasmic reticulum (ER), while $Ins(1,3,4,5)P_4$ has a role in calcium entry, which appears to depend on the action of $Ins(1,4,5)P_3$. Details of the metabolism of inositol phosphates are described in the text.

lyze Ins(1,3,4)P_3 to Ins(3,4)P_2, and Ins(1,4)P_2 to Ins4-P (47), while a lithium-insensitive 4-phosphatase converts Ins(1,3,4)P_3 to Ins(1,3)P_2, and Ins(3, 4)P_2 to Ins3-P (48). Ins(1,4)P_2 forms most of the bisphosphate found in stimulated cells and is further dephosphorylated to Ins4-P. Direct hydrolysis of PI by phospholipase C yields exclusively Ins1-P. All three forms of monophosphates, i.e. Ins1-P, Ins3-P, and Ins4-P, are hydrolyzed by a lithium-sensitive inositol monophosphate phosphatase (51).

A further complexity in the metabolism of inositol phosphates is the demonstration that phospholipase C hydrolyzes all three forms of phosphoinositides into mixtures of cyclic and noncyclic inositol phosphates. Thus, the water soluble products of phospholipase C action on PIP_2 are a mixture of Ins(1,4,5)P_3 and a derivative with the 1-phosphate cyclized between the 1- and 2-hydroxyls termed inositol cyclic 1:2,4,5-trisphosphate or Ins-(c1:2,4,5)P_3 (52). Majerus and coworkers (52) using human platelet and kidney have shown that the cyclic diester bond is not cleaved until the 5- and 4-phosphates are removed, finally producing Ins1-P (53). Ins(c1:2,4,5)P_3 is not phosphorylated by the 3-kinase and is only poorly utilized by the 5-phosphatase (54, 55). In stimulated cells, the cyclic trisphosphate accumulates only after persistent stimulation (56), presumably due to its slow metabolism. Irvine et al (42) found that Ins(1,4,5)P_3 and Ins(cl:2,4,5)P_3 are equipotent in releasing calcium in permeabilized Swiss mouse $3T_3$ cells. Thus, Ins(cl:2,4,5)P_3 is likely involved in some delayed responses induced by stimulation of phospholipase C-coupled receptors, such as proliferation, cell growth, and information storage in the nervous system.

MODE OF ACTION OF DG AND INOSITOL PHOSPHATES

DG: Activation of protein kinase C

This neutral lipid, generated by the hydrolysis of PIP_2, PIP, and PI, functions as a second messenger by activating protein kinase C, as reviewed by Nishizuka (5, 11). This enzyme is a serine/threonine kinase requiring both calcium and phospholipid, preferably phosphatidylserine, for full activity. DG can further activate this enzyme by lowering its calcium requirement such that it is activated even at physiological intracellular concentrations of calcium (0.1 μM). Protein kinase C is also identified as the "receptor" for biologically active phorbol esters, such as tumor-promoters phorbol dibutyrate (PDB) and 12-0-tetradecanoyl phorbol-13-acetate (PMA) that mimic the action of DG by binding to a domain separate from the catalytic site of the enzyme. In the resting state, the kinase is mainly present in the cytosol and is presumably inactive. However, when the cell is stimulated, DG transiently accumulates in the plasma membrane, and protein kinase C is translocated to

the membrane. This kinase C translocation is probably driven by Ins-(1,4,5)P_3-released calcium and serves to prime the enzyme for the activation by DG. This kinase translocation may also in some way increase the availability and subsequent phosphorylation of substrates for protein kinase C, and result in diverse physiological responses. Prolonged activation of protein kinase C leads to down-regulation of this enzyme, presumably due to proteolytic degradation.

Previously, protein kinase C was envisioned as a single enzyme with a widespread distribution in mammalian tissues, but several groups have reported the molecular cloning of multiple forms of its cDNA from a variety of sources (57–61). Huang and colleagues (62) have purified three forms of rat brain kinase isozymes of 82 kD, termed types I–III. Their recent studies using isozyme-specific antibodies to determine the expression products of cells transfected with three forms of cDNA have demonstrated that type I, II, and III protein kinase C are products of protein kinase C genes τ, β and α, respectively (63). Recently, Nishizuka and coworkers (64) have shown that as many as seven distinct types of protein kinase C may exist in rat brain. Although the members of this protein kinase C family share a high degree of sequence homology, they clearly have different regulatory domains (64). There is a need to study tissue distribution, subcellular localization, and substrate specificity of each form of kinase C and their regulation elicited by various external stimuli, to assign the physiological role of each member of the kinase family.

Very little is known about the physiological protein targets of protein kinase C. On the basis of in vitro experiments, Nishizuka (11) listed a group of functionally defined proteins that serve as substrates for protein kinase C. This list grows rapidly, although the physiological meaning of the phosphorylation of these protein substrates remains poorly understood. Among others, both membrane-bound and cytosolic phospholipase C from rat basophilic leukemia cells appear to be phosphorylated by kinase C after treatment of cells with phorbol esters (28). In addition to myosin light chain, a 92.5-kD protein with unknown identity is the substrate for protein kinase C in homogenates of rat aorta smooth muscle cells (65). A putative kinase C substrate, protein F_1, is probably identical to the GAP_{43} protein, the pp46 growth cone protein, and the B-50 protein, as reviewed recently by Benowitz & Routtenberg (66). This protein is linked to axonal growth and may participate in synaptic plasticity and long-term potentiation in the brain hippocampus (see below).

Inositol phosphates: Calcium release and entry

Streb et al (17) first demonstrated that Ins(1,4,5)P_3 releases calcium from a nonmitochondrial store (endoplasmic reticulum) in permeabilized pancreatic

acinar cells. This observation has been confirmed in many other permeabilized cell systems, as reviewed by Berridge (2,12). Dawson et al (67) first showed that the release of sequestered calcium by Ins(1,4,5)P_3 is markedly enhanced if the preparation is preincubated with GTP, but not with related nucleotides, in the presence of the membrane fusogen, polyethylene glycol. A complicating factor is that GTP by itself can also release calcium, even in the absence of $InsP_3$ (67, 68). Such studies led to the hypothesis that $InsP_3$ and GTP release distinct pools of calcium by separate mechanisms. Recently, Thomas (69) used permeabilized hepatocytes to demonstrate that GTP pretreatment can double the magnitude of the Ins(1,4,5)P_3-releasable calcium pool without causing calcium release on its own. Thus, at least in hepatocytes, GTP appears to increase the portion of $InsP_3$-sensitive pool, either by unmasking a cryptic Ins(1,4,5)P_3-sensitive calcium storage pool or by allowing direct calcium flow between $InsP_3$-sensitive and insensitive calcium storage sites. Recent results of Gill and coworkers (70) strongly suggest that GTP does allow calcium to pass between normally distinct endoplasmic reticulum pools. The $InsP_3$-induced release is independent of sodium or calcium (71, 72) and was proposed to utilize a tetraethylammonium-sensitive K^+ current as the counter ion (72). Recently, Meyer et al (73) demonstrated that the rate of calcium release in permeabilized rat basophilic leukemia cells is dependent upon the concentration of added Ins(1,4,5)P_3 in the low nM range and that a calcium "channel" is opened in less than 4 sec by the cooperative binding of more than three molecules of $InsP_3$ to the channel.

A high affinity binding site for Ins(1,4,5)P_3 was first identified in bovine adrenal cortex (74) and subsequently in other tissues, including the brain. In general, binding of radiolabeled Ins(1,4,5)P_3 to the receptor site is rapid and stereospecific, with a rank order of potencies corresponding to that for the release of calcium from microsomes (74–77). The binding site in rat cerebellum is particularly abundant (20 pmol/mg protein) and strongly displays inhibition by calcium (IC_{50} ~ 300 nM) (75, 76). This calcium sensitivity is compatible with the view that calcium released by $InsP_3$ receptor stimulation may serve as negative regulator to turn off further receptor activation. This regulation may also be relevant to the finding that intracellular free calcium concentration oscillates when cells are stimulated with hormones or injected with Ins(1,4,5)P_3 (78). The $InsP_3$ receptor binding activity (76) and $InsP_3$-induced release of calcium from microsomes (71) are sharply increased at slightly alkaline pH. Since intracellular pH is increased by growth factors and hormones that stimulate phosphoinositide metabolism and by phorbol esters that stimulate protein kinase C (3), the effect of pH may reflect augmentation of the ability of $InsP_3$ to release calcium from nonmitochondrial stores after activation of protein kinase C. The level of $InsP_3$ receptors in the brain is at

least 10 times higher than that found in peripheral tissues and parallels the high levels of protein kinase C also found there. Brain autoradiography by Snyder and coworkers (75) showed that in some regions, such as the cerebellar molecular layer and hippocampus, localizations of $InsP_3$ receptors resemble those of protein kinase C, while in others, such as olfactory bulb and substantia gelatinosa, they differ markedly. Thus, relative activity of these two arms of phosphoinositide metabolic cycle can be differentially distributed and regulated. This regional variation in the distribution may serve to confer versatility in the utilization by the brain of the phosphoinositide signal transduction system.

There is a general consensus that activation of phospholipase C-coupled receptors causes a mobilization of intracellular calcium and an entry of extracellular calcium, but the mechanisms underlying this entry are largely unknown. Irvine & Moor (79) provided the first evidence that Ins(1,3,4,5)P_4 may act to promote calcium entry in the presence of an inositol phosphate calcium mobilizer. They found that microinjection of a submicromolar concentration of Ins(1,3,4,5)P_4 into eggs of the sea urchin *Lytechninus variegatus* causes an immediate raising of the fertilization envelope, which requires both the presence of extracellular calcium and coinjection of a stable calcium mobilizer Ins(2,4,5)P_3. These results were interpreted to suggest that Ins(1,3,4,5)P_4 regulates calcium entry across the plasma membrane; however, in some way, the release of the endoplasmic reticulum calcium pool may be a prelude for the action of Ins(1,3,4,5)P_4. An intriguing capacitative model was proposed by Putney (80) to explain the entry of calcium into the cell: calcium entry induced by Ins(1,3,4,5)P_4 is the consequence of a continuous emptying and refilling of the Ins(1,4,5)P_3-sensitive calcium pool following cell stimulation. However, the mechanism by which the $InsP_3$-sensitive calcium pool dictates the entry of extracellular calcium is a mystery. Irvine & Moor (81) recently proposed that Ins(1,3,4,5)P_4, perhaps in concert with GTP, promotes the coupling of endoplasmic reticulum to the plasma membrane, and thus allows a calcium flow from the cell surface into the endoplasmic reticulum, and from there into the cytosol through a Ins(1,4,5)P_3-controlled channel. Putative Ins(1,3,4,5)P_4 binding sites are reportedly enriched in rat brain compared to their peripheral tissues (82). However, potencies of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in displacing the ligand to the putative $InsP_4$ binding sites are virtually identical ($IC_{50} \simeq 300$ nM) (82). In contrast, the apparent affinity of the binding sites in HL-60 cells for Ins(1,3,4,5)P_4 is over 200-fold greater than that for Ins (1,4,5)P_3 (83). Clearly, future biochemical, pharmacological, and physiological studies and ultrastructural localization of $InsP_4$ binding sites are warranted before one can conclude that they are the physiological receptors.

PHARMACOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF PHOSPHOLIPASE C-COUPLED RECEPTORS

The phosphoinositide cascade, which results in mobilization and entry of calcium and activation of protein kinase C, is triggered by activation of phospholipase C–coupled receptors. The development of the methodology by Berridge and colleagues (84) using lithium-dependent accumulation of labeled inositol phosphates to measure phosphoinositide hydrolysis markedly increases the sensitivity of detection and has facilitated the identification of cell-surface receptors coupled to phospholipase C in tissue slices, dissociated cells, and cultured cell lines. At least 20 biologically and pharmacologically active substances have presently been claimed to activate the release of labeled inositol phosphates in intact and broken cell preparation. Table 1

Table 1 Some target tissues in which a stimulus-induced phosphoinositide turnover has been reported

Stimulus (receptor)	Target tissue
Serotonin (5-HT_2)	Most brain regions; primary neuronal cultures of cerebellar granule cells; primary glial cultures; C6-glioma; smooth muscle; platelets; blowfly salivary glands
(5-HT_{1c})	Choroid plexus
Acetycholine (M_1, M_2, M_3)	Most brain regions; primary cultures of astrocytes and of cerebral and cerebellar neurons; neuroblastoma; astrocytoma; neurohybrid NCB-20; superior cervical ganglion; pheochromocytoma; adrenal medulla; heart cells; pancreas; parotids; pituitary tumor; smooth muscle
Norepinephrine (α_1)	Most brain regions; primary cultures of cerebral and cerebellar neurons; smooth muscle; adiopocytes; parotids; vas deferens; pineal glands
Histamine (H_1)	Most brain regions; primary cultures of cerebellar neurons; chromaffin cells; neuroblastoma; astrocytoma; neurohybrid NCB-20
Glutamate	Brain hippocampus and striatum; primary cultures of cerebellar and striatal neurons
Bradykinin	Neurohybrid NG108–15 and NCB-20; primary cultures of cerebellar neurons; neuroblastoma; anterior pituitary cells; smooth muscle; hepatocytes; kidney
Substance P	Brain regions; smooth muscle; parotids
Angiotensin II	Anterior pituitary; smooth muscle; hepatocytes; kidney; pheochromocytoma
Vasopressin (VI)	Brain regions; superior cervical ganglion; smooth muscle; hepatocytes
Neurotensin	Brain regions; neuroblastoma
TRH	Pituitary tumor cells; cerebellar cortex

illustrates some target tissues in which a stimulus-induced phosphoinositide turnover has been documented. Recent advances in the development of specific receptor agonist, antagonists, and radiolabeled ligands have contributed greatly to our understanding of the pharmacology of phospholipase C-coupled receptors. There is compelling evidence that certain receptor subtypes are involved in the phosphoinositide cycle, while others are coupled to other effector systems. The success of gene cloning of some neurotransmitter receptor subtypes has provided a tool to study the relationship between receptor structure and its biological activity. It is also becoming clear that there is "cross-talk" between phospholipase C-coupled receptors and other receptor-mediated effector systems such as adenylate cyclase (see below). In this section, the pharmacological and biochemical characteristics of some major phospholipase C-coupled neurotransmitter and neuropeptide receptors are reviewed.

Serotoninergic Receptors

The first demonstration that stimulation of serotoninergic receptors leads to activation of phosphoinositide turnover was the study of Jafferji & Michell (85) in longitudinal muscle strips of guinea pig ileum. This serotoninergic phosphoinositide response was confirmed by Berridge and coworkers using salivary glands and rat brain (84, 86). Much of the confusion and attention in this area has centered on the classification of serotonin receptors and receptor subtypes involved in phosphoinositide metabolism. At present, pharmacological characterization has divided the receptor into three major subtypes termed 5-HT_1, 5-HT_2, and 5-HT_3; the 5-HT_1 receptor has four different subclasses, i.e. 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} (for review, see 87–89). Its pharmacological relevance began to unfold in 1984, when three groups independently presented evidence that the setotonin responses in vascular smooth muscle (90), cerebral cortex (91), and platelets (92) were inhibited by a 5-HT_2 receptor antagonist ketanserin. Subsequent pharmacological studies support a strong, positive correlation between the rank order of potencies of serotonin antagonists for the 5-HT_2 receptor sites and for the blockade of serotonin-induced inositol lipid breakdown (93–96). In rat aorta, 5-HT_2 receptor-mediated phosphoinositide metabolism is causally linked to serotonin-induced contraction. For instance, Roth et al (93) showed that pretreatment with a phorbol ester attenuates both serotonin-mediated inositol phosphate accumulation and aortic contraction. Nakaki et al (97) demonstrated that the tonic phase of serotonin-induced aortic contraction may involve the activation of protein kinase C by DG, since the action can be mimicked by phorbol ester application. Conversely, the phasic component of aortic contraction may require elevation of intracellular calcium. In these regards, phorbol ester was also shown to induce myosin light chain phos-

phorylation and to cause sustained constriction in sphincter smooth muscle of rabbit iris (98). Moreover, recent ingenious experiments by Walker et al (99), using laser pulse photolysis of caged Ins(1,4,5)P_3 to release Ins(1,4,5)P_3, provide strong evidence that Ins(1,4,5)P_3 is a second messenger involved in smooth muscle contraction. These results suggest that both DG and inositol phosphate arms of phosphoinositide cycle activated by serotonin do participate in smooth muscle contraction. 5-HT_2 -mediated phosphoinositide metabolism is expressed in the primary culture of cerebellar granular cell neurons (100) and astrocytes prepared from several brain regions of newborn rats (101), and in C-6 glioma cells (102), and thus indicate its presence in both neurons and glial cells of the CNS.

The pharmacological profile of phospholipase C-coupled serotonin receptors in the brain hippocampus is unclear. Janowsky et al (103) first showed that the serotonin dose-response curve is bimodal and the effect is inhibited by metergoline, but not by a 5-HT_2 receptor antagonist, mianserin. Conn & Sanders-Bush (94) also reported that the hippocampal serotonin response is less sensitive to ketanserin than that of the cortex. Recent experiments by Mellow et al (104) demonstrated that the bimodal serotonin dose response curve can be abolished by the presence of a moderately high concentration of potassium (15–20 mM) and that this high potassium also markedly enhances the response to serotonin. Moreover, the hippocampal serotonin response was shown to be unaffected by a putative 5-HT_3 receptor antagonist, ICS 205–930. In addition, 2-methyl-5-HT (a putative 5-HT_3 agonist) as well as m-chlorophenylpiperazine (a putative 5-HT_{1B} and 5-HT_{1C} agonist) is very weak in stimulating the response (104). Clearly, there is a need to develop more selective receptor subtype drugs and to gather more pharmacological information before the subtype of the phospholipase C-coupled 5-HT receptors in the hippocampus can be assigned.

Evidence is accumulating that the 5-HT_{1C} receptor in the choroid plexus of rat brain is coupled to phospholipase C (105). The serotonin response in this brain region is characterized by a greater potency (10–fold difference) and efficacy of serotonin and by a relative insensitivity to the antagonist spiroperidol. The rank order of potency of serotonin antagonists for blocking the phosphoinositide response and the 5-HT_{1C} receptor binding appears to be highly correlated. Recently, Lübbert et al (106) have isolated a 5-HT_{1C} receptor cDNA clone from mouse choroid plexus papilloma. Their cloning results demonstrated that the 5-HT_{1C} receptor is probably a single subunit, analogous to muscarinic cholinergic and β-adrenergic receptors. Since 5-HT_{1C} in the choroid plexus is not linked to stimulation or inhibition of adenylate cyclase (107), this cloned receptor may serve as a model to study the relationship between receptor structure and coupling to phospholipase C.

Kendall & Nahorski (108) first demonstrated that chronic treatment with

the antidepressant, imipramine or iprindol, results in a respective 17% and 42% decrease in the binding of ^{3}H-ketanserin to 5-HT_2 receptors and a corresponding loss (approximately 60%) of 5-HT_2 receptor-mediated inositol lipid metabolism in the cerebral cortex. Similar attenuation of the cortical 5-HT_2 receptor response was confirmed after treatment with other antidepressants, i.e. mianserin (95) and desipramine (109). Thus, chronic antidepressant-induced down-regulation of the 5-HT_2 receptor site is accompanied by a subsensitivity of 5-HT_2 receptors. Chemical lesions of serotoninergic nerve endings by the neurotoxin, 5,7-dihydroxytryptamine were found not to change the cortical 5-HT_2 receptor-mediated phosphoinositide response in rats (95) and mice (109), although parallel experiments showed a behavioral (head-twitch) supersensitivity mediated by 5-HT_2 receptor stimulation (109). In contrast, denervation with this serotonin neurotoxin induced a significant supersensitivity for the 5-HT_{1C} -mediated phosphoinositide response in the choroid plexus of rats (110). It appears therefore that 5-HT_{1C}, but not 5-HT_2, receptor-stimulated phosphoinositide metabolism is under the tonic influence of serotonin in the brain. The supersensitivity of 5-HT_2 receptor-mediated behavioral changes, but not phosphoinositide hydrolysis, may suggest that more than one population of 5-HT_2 receptors exist in the brain and that the population coupled to phospholipase C does not receive strong serotoninergic input in vivo. This may be related to its relatively low sensitivity to serotonin in vitro and its localization in part on glial cells (101, 102).

Muscarinic cholinergic receptors

Muscarinic cholinergic receptors coupled to phospholipase C have been demonstrated in numerous systems, especially in the CNS and neural related neural tissues (for review, see 13). This response is blocked by classical muscarinic antagonists such as atropine or scopolamine, but is largely insensitive to nicotinic receptor antagonists such as mecamylamine or hexamethonium. Based on sensitivity to the antagonist pirenzepine, it was suggested that a pirenzepine-sensitive M_1 receptor is coupled to phospholipase C activation, while a pirenzepine-insensitive M_2 receptor is linked to adenylate cyclase inhibition (111, 112). It is becoming clear that this classification is an over-simplification. Pharmacological characterization of this receptor revealed the existence of more than two types of muscarinic receptors in the brain and peripheral tissues (113, 114). Receptor gene cloning studies by Bonner and coworkers (115) demonstrated that there are at least four functional muscarinic receptor genes in rat cerebral cortex, M_1, M_2, M_3 and M_4, which vary significantly in their DNA sequences and regional distribution in the brain and, when expressed in mammalian cells, differ in their sensitivity to pirenzepine. Subtypes M_1, M_3 and M_4 are relatively sensitive to pirenze-

pine and might have been previously classified as M_1 receptors. The sensitivity of muscarinic receptor-mediated phosphoinositide response to pirenzepine varies considerably in different systems. Thus, pirenzepine is a potent inhibitor (Ki=10–30 nM) in most brain regions (112, 116, 117), pituitary tumor AtT-20 cells (118), human SH-SY5Y neuroblastoma (119), neuroblastoma NIE-115 cells (120) and neuroblastoma hybrid NCB-20 cells (121), but is only moderately active (Ki=100–350 nM) in the neostriatum (120), chick heart cells (122), astrocytoma (122), parotid glands (112), human SK-N-SH neuroblastoma (120), sphincter smooth muscle (123), and cerebellar granule cells (124), and is very weak in medulla pons (125). Recently, Fisher & Heacock (126), using selective antagonists for M_1, M_2, and M_3 receptors, concluded that a putative M_3 receptor may be coupled to inositol phosphate release in SK-N-SH neuroblastoma. This awaits confirmation by functional expression of M_3-selective cDNA in mammalian cells. In sphincter smooth muscle, carbachol-induced $InsP_3$ accumulation and smooth muscle contraction can be blocked by a M_2 cardioselective antagonist AF-DX116 (123). Similar to this finding, it was shown that the recombinant M_2 muscarinic receptor gene expressed in transfected cells lacking endogenous receptor can be coupled to both adenylate cyclase and phospholipase C (127). One can conclude that, whereas M_2 receptors appear to be coupled to adenylate cyclase, M_1, M_2 and M_3 receptors may be involved in the phosphoinositide cycle.

In general, there is a close relationship between the degree of occupancy of muscarinic receptors by agonists and the extent of stimulation of phosphoinositide hydrolysis. The ability of muscarinic receptor agonists to stimulate phospholipase C correlates well with their potency in displacing the binding of labelled muscarinic ligand to receptor (128). In most systems studied, there is evidence that little receptor reserve exists for phospholipase C-coupled muscarinic receptors. Thus, their inactivation in guinea pig cortex and hippocampus and in neuroblastoma NIE-115 cells by alkylation with propylbenzilylcholine mustard results in reduction of carbachol-induced inositol phosphate accumulation that is equal to, or greater than, the loss of receptor binding sites (120). In chick heart cells, measurements of carbachol-induced phosphoinositide turnover and inhibition of cyclic AMP formation before and after removal of muscarinic receptors by alkylation indicate that there is much greater receptor reserve in the coupling of muscarinic receptors to adenylate cyclase than to phospholipase C (129). This difference in the receptor reserve appears to account for the much greater potency of carbachol for inhibiting cyclase than for stimulating phospholipase C. A similar conclusion was drawn by Zhu & Chuang (130) in recent studies of muscarinic receptors in cultured NCB-20 (neurblastoma x brain cell hybrid) cells. Muscarinic receptors in this cell line can be up-regulated (about 100%) and down-regulated (about 50%)

by long-term treatment with butyrate and dibutyryl cAMP, respectively. These up- and down-regulations are associated with roughly proportional changes in the carbachol-mediated inositol phosphate accumulation without affecting the carbachol EC_{50} values (130). Conversely, there is also indication that a population of "spare" receptors is present for muscarinic receptor-linked phosphoinositide hydrolysis in some systems. In both the guinea pig neostriatum and neuroblastoma SK-N-SH cells, receptor alkylation results in a greater loss of receptor number than of stimulated inositol phosphate accumulation and a significant increase in the EC_{50} value of carbachol (120). Consistent with this interpretation, in neostriatum carbachol was found to be about 20–fold more potent for stimulating phosphoinositide breakdown and partial agonists markedly more effective than in the cerebral cortex (120). Recent studies by Balduini et al (131) and Rooney & Nahorski (132) showed that muscarinic receptor-coupled phosphoinositide hydrolysis in rat cerebral cortex displays a distinct developmental profile, increasing within the first week after birth and then gradually declining during the course of maturation. In contrast, muscarinic receptor number increases throughout development. The dissociation of the number of receptor binding sites from the magnitude of receptor-mediated inositol lipid metabolism may reflect a more efficient coupling at specific times during neonatal development, in particular about one week after birth.

α_1 -Adrenergic Receptors

There is strong evidence that the α_1 -adrenergic receptor subtype is linked to stimulation of inositol phospholipid metabolism. Norepinephrine-induced inositol phosphate release from phosphoinositides can be completely blocked by the α_1 -adrenergic receptor antagonist, prazosin, but the release is virtually unaffected by the α_2 -receptor antagonist yohimbine (133–135). Norepinephrine and epinephrine are full agonists for this α_1 -receptor-mediated response, but phenylephrine and methoxamine are partial agonists, and oxymetazoline and ephedrine are essentially inactive (133–135). As in the muscarinic receptor, there is a close relationship between agonist occupation of α_1 -adrenergic receptors and the activation of phosphoinositide hydrolysis (135, 136). Inactivation of rat brain cortical α_1 -adrenergic receptors with the alkylating antagonist phenoxybenzamine results in a good correlation between the loss of ^{3}H-prazosin binding sites and the decrease in α_1 -receptor-mediated inositol lipid hydrolysis (135). A similar conclusion was made in BC3H muscle cells using phenoxybenzamine inactivation and pseudoinactivation of the receptor by prazosin (136). These observations suggest that there is little reserve of α_1 -adrenergic receptors coupled to phospholipase C in the normal state.

Studies of the effects of noradrenergic denervation on the α_1 -adrenergic receptor-mediated phosphoinositide response have generated controversy. Evidence both for and against the changes in the α_1 -adrenergic response is available, and the nature of reported changes varies greatly among systems studied. Janowsky et al (137) first demonstrated that the maximal extent, but not the EC_{50}, of norepinephrine-induced inositol phosphate accumulation in hippocampal slices is markedly enhanced following intracerebroventricular administration of 6-hydroxydopamine or unilateral surgical interruption of the ascending medial forebrain bundle. Subsequent studies by Fowler et al (138) found no change in the α_1 -adrenergic response following noradrenergic denervation in a similar hippocampal preparation. Recently, Nicoletti et al (139) showed that lesioning of rat hippocampal neurons by intrahippocampal infusion of kainate or colchicine increases the phosphoinositide response in hippocampal slices elicited by saturating concentrations of norepinephrine, while unilateral decortication enhances the maximal norepinephrine response in the striatal preparation. These lesions also potentiate the response mediated by some excitatory amino acid receptors (see below) but fail to affect the response stimulated by muscarinic receptors. Kendall et al (135) reported a left shift in the dose response curve of norepinephrine and an increase in the efficacy of the partial agonist phenylephrine in rat cerebral cortex after 6-hydroxydopamine lesions. Using rabbit iris dilator smooth muscle, Akhtar & Abdel-Latif (140) showed that surgical denervation markedly increases the potency of norepinephrine for inducing $InsP_3$ accumulation and muscle contraction. Recent experiments of Minneman et al (141) demonstrated that surgical denervation of α_1 -adrenergic receptors in rat vas deferens increases 10–fold the potency of norepinephrine and 3.5–fold that of epinephrine in stimulating phosphoinositide hydrolysis. The disparity of these results may stem from differences in the methodology of pharmacological manipulations, in conditions of in vitro assays for inositol phosphate release, in age and species of experimental animals, and in tissues and preparations used by various laboratories. The development of a reliable in vivo method for measuring the turnover of phosphoinositides following receptor agonist stimulation may help resolve some of these discrepancies.

Abnormalities in the level of α_1 -adrenergic receptor binding sites and α_1 receptor-mediated phosphoinositide response have been reported in animal models for disease states. In a neuronal preparation derived from hypertensive rats, the density of α_1 -adrenergic receptor sites is 30–45% higher, when compared to normotensive controls (142). Paradoxically, the α_1 -adrenergic receptor-stimulated inositol phosphate release is 40% less responsive in neurons of hypertensive rats than in the controls, while the muscarinic receptor response was unchanged (142). It is possible that an elevated level of α_1 -adrenergic receptor triggers a compensatory mechanism to suppress its

effector response, and thereby maintains a homeostasis of the receptor activity. In the brain of genetically epilepsy-prone rats, the density of ^{3}H-prazosin binding sites and the maximal stimulation of norepinephrine-induced inositol phosphate accumulation are markedly decreased in the frontal cortex, but not in other brain regions, including the hippocampus, corpus striatum, and inferior colliculus (143); this suggests that a deficit in the α_1 -adrenergic receptor system in the frontal cortex may play a role in the seizure process in these animals. Ethanol at concentrations as low as 100 mM was reported to inhibit noncompetitively the hydrolysis of phosphoinositide elicited by norepinephrine and glutamate in several brain regions studied, while the responses to carbachol and serotonin were relatively less affected (144). It seems likely that alteration of the α_1 -adrenergic receptor-coupled phospholipase C activity may contribute to some of the pharmacological effects of alcohol.

Excitatory Amino Acid Receptors

Excitatory amino acids, primarily L-glutamate and L-aspartate, are major neurotransmitters in the CNS. Electrophysiological and pharmacological characterization has divided the receptors for excitatory amino acids into at least three distinct systems, i.e. N-methyl-D-aspartate (NMDA), quisqualate, and kainate. Nicoletti et al (145) demonstrated that ibotenic acid, a rigid heterocyclic analogue of glutamic acid, induces a large increase in inositol phosphate formation in hippocampal slices of adult rats. This ibotenate response is inhibited by amino-4-phosphonobutyric acid, and the pharmacological profile of this response is inconsistent with any of above three classes of receptors, but resembles the fourth putative excitatory receptor (amino-4-phosphonobutyrate sensitive site) described by Monaghan et al (146). Glutamate and aspartate do not substantially activate phosphoinositide breakdown in hippocampal slices of adult rats, but they markedly stimulate the inositol lipid metabolism in the hippocampus of newborn rats (147). The magnitude of this response declines progressively during postnatal development and is essentially absent after day 24 of life. Using dissociated striatal neurons of 14-day-old mouse embryos, Sladeczek et al (148) also reported that excitatory amino acids induce a large increase in $InsP_1$, $InsP_2$ and $InsP_3$ formation with the relative potency of quisqualate > glutamate > NMDA, kainate. The glutamate-evoked formation of inositol phosphates is compatible with the activation primarily of quisqualate, and to a lesser extent, kainate receptors. Recently, it was found that in striatal neurons, stimulation of NMDA and kainate receptors leads to attenuation of phosphoinositide turnover elicited by muscarinic cholinergic receptor stimulation with carbachol, while the effects of quisqualate and carbachol are additive (149). The inhibition of the carbachol response by NMDA appears to be due, at least in

part, to the neurotoxicity of NMDA on the cholinergic neuron, as revealed by enhanced release of lactate dehydrogenase following NMDA exposure. Costa and coworkers (139) have provided evidence that lesions of putative glutamatergic pathways potentiate the phosphoinositide response elicited by excitatory amino acids. The pharmacological manipulatons used in their studies are intrahippocampal infusion of kainate that preferentially destroys neurons in the CA_{3-4} areas, infusion of colchicine that selectively destroys granule cells of fascia dentata, and ablation of the frontal cortex that removes the glutamatergic afferents to the striatum. All three types of lesions markedly potentiate the responses of ibotenate, glutamate, and quisqualate in their target tissue, i.e. hippocampus or striatum, while NMDA remains ineffective in promoting the response, and thus indicates selective modulation of some subtypes of excitatory amino acid receptors.

A primary culture of cerebellar granule cell neurons prepared from eight-day rats also expresses excitatory amino acid receptors coupled to phospholipase C (150). Excitatory amino acids enhance the inositol phospholipid metabolism with a potency of glutamate > quisqualate > kainate > aspartate > NMDA, ibotenate. The action of glutamate and aspartate is preferentially inhibited by 2-amino-5-phosphonovalerate, a selective NMDA receptor antagonist, while this antagonist does not affect, and only marginally inhibits, the responses mediated by quisqualate and kainate, respectively. The response of NMDA receptor activation in granule cells is attenuated by the presence of Mg^{2+} and is noncompetitively inhibited by phencyclidine and its congeners (151). Phencyclidine also inhibits Ca^{2+} influx and cGMP production mediated by Mg^{2+}-sensitive NMDA receptors (151). The potency of glutamate, aspartate, and NMDA for inducing the phencyclidine and Mg^{2+}-sensitive Ca^{2+} influx can be enhanced by the presence of glycine (152). These observations support the notion that the NMDA receptor subtype possesses multiple interacting regulatory components and is perhaps even more complex than the γ-aminobutyric acid/Cl^- ionophore/benzodiazepine receptor complex to which it bears some similarities. The recent success of the expression of NMDA receptors in *Xenopus* oocytes injected with rat brain messenger RNA (153) may serve as a tool for molecular cloning of the receptor gene, as has been successfully used for the cloning of $5\text{-}HT_{1C}$ receptors (106). Stimulation of cultured cerebellar neurons with glutamate leads to a rapid translocation of protein kinase C from the cytosol to the membrane, as revealed by binding of ^{3}H-phorbol dibutyrate to intact cells. This kinase C translocation in granular cells can also be demonstrated by immunoblotting using antibody specific for type II or III kinase C isozymes (F. L. Huang, K. P. Huang, D.-M. Chuang, unpublished observations). This enzyme translocation can be inhibited by the gangliosides GT_{1b} and GM_1 (154), an event most likely due to intercalation of these compounds into the plasma membrane, and their subsequent interference with the association of

kinase C with the membrane phosphatidylserine. This effect could be related to the ability of gangliosides to protect neurons in culture from glutamate intoxication (155).

Excitatory amino acid receptors have been implicated in the learning and memory processes. For example, the density of NMDA receptor binding sites is markedly enhanced in the chick forebrain during the learning process of imprinting (156). Long-term potentiation (LTP) of synaptic transmission induced by high-frequency stimulation has been used as a model of memory and learning. LTP in the hippocampus has been shown to be blocked by aminophosphonobutyric acid (157) and NMDA receptor antagonists (158). Recent experiments showed that LTP in the hippocampus is associated with translocation of protein kinase C to the membrane (159) and a corresponding increase in the phosphorylation of the protein kinase C substrate, protein F_1 (66). Hippocampal LTP was eliminated by protein kinase C inhibitors (mellitin, polymyxin B, H-7) applied before or shortly after LTP (160). Recently, intracellular injections of protein kinase C into hippocampal neurons (161) or the protein kinase C activator, phorbol dibutyrate into neocortical neurons (162) were shown to elicit features of LTP. In addition, in the post-mortem brain of patients with Alzheimer's disease, the NMDA receptor sites decline in response to neuronal loss and the glutamatergic neurotransmission within the hippocampal formation is severely impaired (163, 164). Thus, glutamate-induced activation of phosphoinositide turnover and corresponding protein kinase C activation and translocation in some brain areas may be involved in the processes of learning and memory.

Peptidergic Receptors

Receptor-mediated phosphoinositide hydrolysis has been observed after stimulation by a number of peptides, including bradykinin, thyrotropin-releasing hormone (TRH), substance P, bombesin, cholecystokinin, neurotensin, vasopressin, and angiotensin. Among these, the bradykinin-induced inositol lipid breakdown has attracted the most attention because electrophysiological studies of this receptor-mediated event elegantly demonstrated the involvement of phosphoinositide metabolites in neural transmission. Higashida & Brown (165) showed that application of bradykinin to neuroblastoma x glioma hybrid NG108–15 cells triggers a transient hyperpolarization that is followed by depolarization lasting for approximately one minute. The initial hyperpolarization can be reproduced by intracellular injection of calcium or Ins(1,4,5)P_3, but not by Ins(1,3,4)P_3 or Ins (1,3,4,5)P_4; this is caused, at least in part, by the opening of a calcium-dependent potassium channel. In contrast, the depolarization appears to be mediated primarily by activation of the DG/protein kinase C pathway and involves the closing of a different voltage-sensitive potassium channel (M

current). The temporal dissociation of the transient hyperpolarization from the longer-lasting depolarization may be explained if DG is derived not only from the initial PIP_2 breakdown, but also from subsequent PI and PIP_2 hydrolysis (see preceding section on hydrolysis of phosphoinositide). DG may also be formed by cleavage of phosphatidylcholine after stimulation of calcium-mobilizing receptors, as suggested by Exton and coworkers (166). This would provide an independent regulation of $InsP_3$ and DG synthesis and a differential activation of the calcium and protein kinase C-linked second messenger systems. It is interesting that a phorbol ester markedly potentiates the outward current elicited by $InsP_3$ application (167), in contrast with predicted facilitation of Ins(1,4,5)P_3's removal by protein kinase C activation and the known inhibitory effect of phorbol esters on receptor-mediated activation of phospholipase C (see below). This observation may reflect a strong positive feed-back effect of bradykinin-induced kinase C activation on the sensitivity of a potassium current to calcium that outweighs any inhibitory effect in NG108-15 cells. In other neural systems, inhibition of M current by stimulation with substance P, serotonin, and acetylcholine is associated with depolarization, whereas opening of this potassium channel by somatostatin is accompanied by hyperpolarization (for review, see 168). These studies suggest that regulation of this potassium channel by the phosphoinositide mechanism may play a fundamental role in controlling some synaptic transmission.

REGULATION OF PHOSPHOLIPASE C-COUPLED RECEPTORS

Role of GTP Binding Protein

The first evidence that a GTP binding protein (G protein) is involved in phospholipase C-catalyzed phosphoinositide hydrolysis came from studies using permeabilized cells. Gomperts (169) reported that guanine nucleotides stimulate a calcium-dependent histamine secretion from permeabilized mast cells and, therefore, proposed that regulatory G proteins participate in the secretory process triggered by phosphoinositide hydrolysis. Litosch et al (170), using a cell-free system from blowfly salivary glands, demonstrated that nonhydrolyzable GTP analogues, Gpp(NH)p and GTPγS, stimulate the production of inositol phosphates from endogenously labeled phosphoinositides and that this effect is potentiated by the presence of serotonin. Similar observations on the effects of guanine nucleotides and their potentiation of hormonal responses in a membrane preparation have been extended to other systems, including neutrophils, polymorphonuclear leukocytes, cerebral cortex, hepatocytes, GH_3 cells, and coronary artery, as reviewed previously (171). In the plasma membrane of rat liver (172) and human polymorphonuclear leukocytes (173), GTP analogues and receptor agonists were found to

activate the hydrolysis of labeled PIP_2 mainly by lowering its calcium requirement, which suggests that the site of calcium action may be at the level of G protein. However, other investigators have reported that guanine nucleotides induce an additive effect on the stimulation elicited by calcium in the cerebral cortical membranes (174, 175) and cause a synergistic effect on calcium-induced activation in rat aortic membranes (29) and permeabilized chick heart cells (176). Recent experiments of Gonzales & Crews (177) demonstrated that in cerebral cortical membranes GppNHp− and calcium-stimulated phosphoinositide breakdowns have distinct pH optima and sensitivities to neomycin and chloroquine, results that suggest either the existence of two separate species of phospholipase C or an involvement of two distinct regulatory mechanisms. Whether this phenomenon can be found in a more homogenous cell population such as the liver or clonal cell lines awaits confirmation.

The identity of the G protein coupled to phospholipase C remains unknown. In platelet (178), neutrophils (179), mast cells (180), adipocytes (181), leukemic cells (182), and neurohybrid NCB-20 cells (183), pretreatment with pertussis toxin completely or partially abolishes phospholipase C activation induced by agonists for calcium-mobilizing receptors. In the majority of instances this pretreatment does not affect the receptor-mediated event. Pertussis toxin catalyzes ADP-ribosylation of the α_i subunit of G_i protein linked to inhibition of adenylate cyclase, and this covalent modification abolishes the ability of G_i to inhibit the cyclase. In the case of platelets, some evidence suggests that a single 41–kD protein (α_i) ADP-ribosylated by pertussis toxin may be involved in both the stimulation of phospholipase C and inhibition of adenylate cyclase (178). Crouch & Lapetina (184) also presented evidence that the α_i of G_i in platelets is necessary in the coupling of thrombin receptors to phospholipase C and that this process is uncoupled by phosphorylation of α_i by activated protein kinase C. In either case, there is no compelling evidence that α_i is indeed directly coupled to phospholipase C and its corresponding receptors. Thus, many of the inhibitory effects observed with pertussis toxin might be due to the perturbation of cAMP levels resulting from prolonged α_i inactivation, or the consequence of ADP-ribosylation of G proteins distinct from the G_i. The latter possibility is further suggested by recent reports that novel pertussis toxin substrates with G protein-like properties were found in neutrophils (185), human erythrocytes (186), and leukemic cells (187). Moreover, inositol phosphate formation stimulated by arg-vasopressin in smooth muscle cells (188), and by cholecystokinin and acetylcholine in pituitary clonal cells (189), can be inhibited by prolonged pretreatment with cholera toxin, which ADP-ribosylates the α_s of the G_s protein coupled to adenylate cyclase. In the case of pituitary cells (189), the inhibition by cholera toxin is not reproduced by treating cells with the β-subunit of cholera toxin or cAMP-generating agents such as forskolin—an observation

indicating additional complexity of the phospholipase-coupled G protein. In any event, it is becoming apparent that various calcium-mobilizing receptors present in the same cell preparation can involve distinct G proteins that are differentially regulated. For example, in platelets, pertussis toxin selectively inhibits phosphoinositide breakdown stimulated by thrombin, but not that stimulated by U 46619, a thromboxane A_2 analogue (178). In hepatocytes, the synthesis of Ins(1,4,5)P_3 stimulated by epidermal growth factor or angiotensin II is differentially inhibited by pertussis toxin and phorbol esters (190).

Effect of Sodium Channel Activators

Daly and coworkers (191–193), using brain synaptoneurosomes, first demonstrated that agents activating voltage-sensitive sodium channels, such as batrachotoxin (BTX) and veratridine, stimulate the release of inositol phosphates from phosphoinositides. Similar observations using related drugs have been extended to the cardiac system (176, 194) and clonal cell line NCB-20 (195). These effects were largely dependent on the presence of calcium in the extracellular medium (176, 191, 194, 195), although mechanisms underlying this activation remain unclear. In the case of veratridine-induced inositol phosphate formation in brain synaptoneurosomes (191) and chick heart (176), depletion of extracellular sodium was reported to cause a partial and complete inhibition of the veratridine effect, respectively; this suggests possible involvement of the Na^+/Ca^{2+} exchange mechanism. However, in NCB-20 cells (195) the degree of stimulation induced by BTX and veratridine is not decreased by removal of extracellular sodium, and indicates that the influx of Na^+ is not a prerequisite for the phospholipase C activation. Since a G protein has been proposed to regulate sodium channel activity (196), and compelling evidence supports the coupling of a G protein to phospholipase C (see the preceding section), it is possible that conformational changes induced by sodium channel agonists may directly or indirectly increase the availability of a G protein for membrane-bound phospholipase C. In rat cerebral cortical and atrial slices, the carbachol-induced stimulation of inositol phosphate release is less than additive with the effect produced by BTX (194) and is partially inhibited by a sodium channel blocker, tetradotoxin (194). Muscarinic receptor-mediated phosphoinositide hydrolysis in these preparations may occur by dual pathways and one of these may be depolarization-dependent, involving sodium channel gating. However, such a carbachol effect was not seen in clonal NCB-20 cells (D.-M. Chuang, unpublished observations). Therefore, complex interactions between different cell types in the brain and heart may be required for the multiple pathways to be evident.

Desensitization of Calcium-Mobilizing Receptors

Persistent stimulation with agonists for calcium-mobilizing receptors leads to desensitization of the phosphoinositide response to subsequent stimulation.

This phenomenon has been demonstrated in a number of systems, including astrocytoma cells (197, 198), primary cultures of cerebellar granular cells (124, 199), and cerebral neurons (200), smooth muscle cells (201), and parotid acinar cells (202, 203). The time course and nature of this agonist-induced receptor desensitization vary greatly between systems. The desensitization of α_1 -adrenergic receptors in smooth muscle cells (201) and substance P receptors in acinar cells (202, 203) occurs within minutes after exposure to their respective agonists. Conversely, the desensitization of muscarinic receptors in astrocytomas (197) and cerebellar granular cells (124, 199) requires much longer exposure to the agonist carbachol. Substance P-induced desensitization of $InsP_3$ formation in acinar cells is temporally correlated with the loss of substance P-induced calcium mobilization (203). In contrast, carbachol-induced desensitization of receptor-mediated calcium efflux occurs without concomitant desensitization of phosphoinositide hydrolysis (197). The latter observation suggests that desensitization of the calcium-mobilizing response is distal to the receptor-G protein-phospholipase C complex and may be at the level, for instance, of receptors for Ins(1, 4, 5)P_3 and/or Ins(1,3,4,5)P_4. Homologous desensitization of phospholipase C-coupled muscarinic, α_1 -adrenergic, serotoninergic, and histaminergic (H_1) receptors has been demonstrated by Dillon-Carter & Chuang (199) using primary culture of cerebellar granular cell neurons preexposed to their respective agonists for 30 min to 18 hr. Similarly, substance P-induced desensitization of the substance P response in acinar cells (202, 203) and norepinephrine-induced desensitization of the α_1 -adrenergic effect in cultured neurons (200) does not cross-desensitize with the phosphoinositide response mediated by carbachol. However, heterologous desensitization of calcium-mobilizing receptors has also been well documented. In smooth muscle cells, pretreatment with bradykinin induces desensitization to both bradykinin and norepinephrine (201). Platelets preexposed to thrombin are desensitized to both thrombin and platelet-activating factor (PAF), although PAF pretreatment selectively desensitizes the PAF-induced $InsP_3$ formation (204). The cross-desensitization of these agonist responses suggests that the regulation occurs at a common step, possibly involving a shared effector molecule such as G protein or phospholipase C. In astrocytoma, carbachol- and histamine-induced calcium mobilizations show heterologous desensitization, which suggests that carbachol and histamine mobilize and deplete the same intracellular calcium store during the process of desensitization (198).

Receptor down-regulation is often associated with desensitization of phospholipase C-coupled receptors; however, neither the mechanism of receptor loss nor its role in desensitization is clear. Using cerebellar granular cell culture, Xu & Chuang (124) demonstrated that desensitization of muscarinic receptors can be dissected into fast and slow components, and only the slow component (> 2 hours) appears to be associated with the loss of muscarinic

receptors. Moreover, the time course and extent of receptor loss assessed by using a hydrophilic ligand ^{3}H-N-methylscopolamine, or ^{3}H-NMS, and a lipophilic ligand ^{3}H-QNB to intact cells were similar (124). This is in contrast to the results of Liles et al (205) who showed that in neuroblastoma NIE-115 cells exposed to carbachol the loss of ^{3}H-NMS binding sites precedes the down-regulation of total muscarinic receptors labeled by ^{3}H-QNB; the internalization of cell-surface receptors may therefore be the event leading to receptor loss. Internalization of receptors may occur during desensitization, but this process involves multiple discrete intermediate steps, only some of which are detected by the method of specific ligand binding to receptors on the whole cell. Reversible loss of substance P receptors in desensitized acinar cells (202, 203) and rapid decrease of surface α_1 -adrenergic receptor number in norepinephrine-desensitized smooth muscle cells (201) also suggest the occurrence of internalization. Phosphorylation of α_1 -adrenergic receptors appears to be intimately coupled to the receptor desensitization in smooth muscle cells (201), although the kinase involved is unknown. Labarca et al (206) first documented that phorbol esters inhibit agonist-induced inositol phosphate accumulation in hippocampal slices. This phenomenon has been replicated in numerous systems of calcium-mobilizing receptors and has been interpreted as a negative feed-back regulation of the receptor response by protein kinase C activation and subsequent phosphorylation of target proteins (e.g. receptor, G protein, and phospholipase C). However, extrapolation of this interpretation for agonist-induced receptor desensitization and down-regulation should be exercised with caution. Unlike the natural activator DG, phorbol esters are metabolically stable and very permeable to the cell. Moreover, there is no evidence at this time that protein kinase C is the sole target for phorbol esters. Therefore, effects produced by phorbol esters might not reflect a naturally occurring desensitization process. Future experiments using more selective activators and inhibitors for protein kinase C may provide insight into the role of this kinase in agonist-induced receptor desensitization.

Inter-Regulation Between Receptors Coupled to Phospholipase C and Other Effectors

The observations that activation of phospholipase C-coupled receptors can either potentiate or inhibit the response mediated by adenylate cyclase-coupled receptors have been reviewed by Nishizuka (5, 11). Since phorbol esters can almost fully mimic agonists for phospholipase C-coupled receptors in producing these effects, it has been suggested that the DG/protein kinase C arm of phosphoinositide metabolism is mainly involved in this regulation. However, in guinea pig cerebral cortical slices, the potentiation of adenosine-stimulated cAMP accumulation by histamine (through H_1 receptor-mediated

phosphoinositide turnover) cannot be replaced by phorbol esters at relatively low doses (1–100 nM) sufficient to activate protein kinase C (207). Recently, Garbarg & Schwartz (208) reported that in guinea pig brain slices, potentiation of histamine H_2 receptor-mediated cAMP accumulation by H_1 receptor stimulation is additive to that produced by phorbol esters. Moreover, the H_1 receptor, but not phorbol ester-mediated stimulation depends on extracellular calcium (208), and therefore may involve the inositol phosphate arm of phosphoinositide cycle in this receptor cross-talk.

Increasing evidence indicates that signal transduction mediated by calcium-mobilizing receptors is subjected to regulation by stimulation of other classes of receptors. Vasoactive intestinal peptide (VIP) has been shown to coexist with acetylcholine in cortical neurons of the CNS (209). In rat cerebral cortical slices, very low concentration (1 nM) of VIP appears to enhance the potency of carbachol for inducing inositol phosphate accumulation (210); however, this preliminary result awaits confirmation. Using brain hippocampal slices, Pepeu and coworkers (211, 212) reported that a γ-aminobutyric $acid_A$ ($GABA_A$) receptor agonist that per se is unable to activate phosphoinositide hydrolysis, enhances the potency of norepinephrine for this inositol lipid breakdown. It is unclear whether this cross-talk is due to direct receptor-receptor interaction or is the result of transynaptic events. Using primary culture of cerebellar granular cells with more than 90% purity, Yu & Chuang (213) recently demonstrated that long-term exposure of these cultured neurons to GABA markedly enhances the maximal extents of the phosphoinositide response mediated by excitatory amino acids such as glutamate, NMDA, and quisqualate, but not kainate. Excitatory transmitters such as glutamate and norepinephrine are known to be involved in long-term potentiation (158, 214). Therefore, the enhancement by GABA of their responses might have physiological significance in the process of memory. Stimulation of the apparent A_1 adenosine receptor enhances histamine, but not norepinephrine, carbachol, 5-HT, or high K^+-elicited formation of inositol phosphate in guinea pig cerebral cortical slices (215, 216). However, in different tissue preparations, i.e. rat striatal (217) and mouse cortical (218) slices, adenosine receptor agonists selectively inhibit the histamine-stimulated inositol lipid hydrolysis with a marked decrease in the maximal effect. Adenosine has been reported also to inhibit TRH-stimulated phosphoinositide hydrolysis in GH_3 cells via a pertussis toxin-sensitive mechanism (219). The disparity of its stimulatory and inhibitory effects could be mutually exclusive events that are species and perhaps cell type specific. Alternatively, both responses may coexist in the same tissue or cell and its apparent action in a particular preparation may be the sum of opposing effects. The D_2 dopamine receptor is another potential candidate that may serve as a negative regulator for calcium-mobilizing receptors. In cerebral striatal slices, various D_2 dopamine receptor

agonists decrease the content of $InsP_3$, and this effect is blocked by a D_2 receptor antagonist, domperidone (220). The dopamine receptor is also involved in the attenuation of TRH- and angiotensin II-stimulated inositol phospholipid breakdown in anterior pituitary cells (221, 222). A preliminary report suggests that 5-HT_{1A} receptor agonists may selectively inhibit carbachol-induced phosphoinositide hydrolysis in the rat hippocampus (223). In summary, although evidence is emerging that calcium-mobilizing receptors can be positively or negatively modulated by stimulation of another class of cell-surface receptors, it is far from clear whether this represents receptor-receptor interactions, as for GABA/benzodiazepine or NMDA/glycine receptor complexes. Since intact cells were used in these studies, much of the effect could be the consequence of interactions between different second messenger systems. Future studies on these interregulations using broken cell preparations are required to address the molecular mechanisms underlying this receptor modulation.

The Action of Lithium

Lithium has been used in the treatment for manic depressive illness for more than three decades, but only recently has its possible mode of therapeutic action begun to emerge. Pioneer studies by Allison, Sherman and coworkers demonstrated that administration of a therapeutic dose of lithium causes a modest decrease in the content of myo-inositol (224). This was later found to be associated with accumulation of inositol monophosphate due to inhibition of inositol monophosphatase (225, 226). Based on these observations, Berridge and coworkers (84), and more recently Drummond (227), proposed that the etiology of manic depressive illness is the consequence of uncontrollable hyperactivity of phosphoinositide metabolism and that lithium, by attenuating the level of inositol, limits the synthesis of phosphoinositide and compromises the generation of second messengers by phospholipase C. This hypothesis is attractive because the level of inositol in the brain is critically dependent on its de novo synthesis, unlike in the periphery where it is readily accessible to dietary inositol. Thus, lithium administration would selectively attenuate the availability of phosphoinositides for phospholipase C in the brain tissue. However, there is no compelling evidence at this time that the level of PIP_2, is decreased after short- or long-term lithium treatment. For example, in GH_3 cells the level of PIP_2 is unchanged by treatment with lithium combined with TRH, despite a significant decrease of PI and PIP contents (228). In parotid glands stimulated with carbachol, lithium reduces the rate of PIP but not PIP_2 biosynthesis (229). The brain contents of PI, PIP, and PIP_2 are partially depleted in rats treated with lithium only under extreme conditions of seizure induced by pilocarpine (230). The observed decrease in the level of PIP and PIP_2 could be due to a decline in ATP content known to

be associated with seizure. It has become evident that lithium has additional sites of action in the inositol phosphate metabolism cycle. Besides inhibiting the action of inositol monophosphatase on Ins1-P_1, Ins4-P_1 and Ins3-P_1, it also blocks the hydrolysis of Ins(1,3,4)P_3 and Ins(1,4)P_2 by inositol polyphosphate 1-phosphatase in the brain (see the preceding section on metabolism of inositol phosphates). In addition, Batty & Nahorski (231) have recently demonstrated that lithium inhibits muscarinic receptor-stimulated accumulation of InsP_4, which plays a role in calcium entry. These additional effects of lithium might contribute to its therapeutic action.

Evidence is emerging that acute and chronic lithium treatment can attenuate phosphoinositide metabolism activated by neurotransmitter receptors. Therapeutic concentrations of lithium were shown to retard the relaxation of smooth muscle elicited by cholinergic or histaminergic stimulation of phospholipase C (232) and to block the phosphoinositide-mediated cholinergic response in hippocampal slices (233). In rat cerebellar cortex, the effects on phosphoinositide hydrolysis mediated by carbachol, histamine, or 5-HT are decreased after acute or chronic lithium treatment (234), while the norepinephrine-induced response is decreased only after chronic administration (234, 235). Neither treatment significantly affects the incorporation of ^{3}H-inositol into phospholipid (234). These effects cannot be readily explained by the classical site of lithium's action at inositol monophosphatase, but they suggest that lithium induces accumulation of phosphoinositide metabolites that then serve as negative regulators for these receptor events. In this context, it is worth noting that lithium induces DG accumulation in GH_3 cells stimulated with TRH (228). If this is a widespread effect, then DG, like phorbol ester, may attenuate receptor-coupled inositol lipid breakdown by activation of protein kinase C and subsequent phosphorylation of target protein at the level of receptor-G protein-phospholipase C complex. Such feed-back regulation involving protein kinase C might also explain the recent finding that lithium inhibits both adrenergic and cholinergic agonist-induced enhancement of GTP binding to G protein coupled to adenylate cyclase (236). Regardless of the mechanisms involved, the inhibition by lithium of various phospholipase C- and adenylate cyclase-linked neurotransmissions may serve to normalize the hyperactivity of neurons proposed to be associated with manic depressive illness. It is tempting to propose that different effector-coupled neurotransmitter systems may be separately involved in manic and depressive episodes and that normalization of individual systems is the pharmacological basis for both the antimanic and antidepressant effect of lithium.

CONCLUSION

The past five or six years have witnessed an enormous growth in the study of phosphoinositide turnover. It is now clear that stimulation of cell-surface

receptors leads to hydrolysis of PIP_2 by phospholipase C and that the dual messenger pathway involving $Ins(1,4,5)P_3$ and DG has evolved as a mechanism designed to control an array of physiological processes. The metabolism of phosphoinositides is proving exceedingly complex. Over 20 inositol phosphate metabolites have been discovered, and the number will undoubtedly continue to grow. Increasing evidence suggests that $Ins(1,3,4,5)P_4$ influences the entry of extracellular calcium with a mechanism dependent upon calcium mobilization induced by $Ins(1,4,5)P_3$. The receptor for $Ins(1,4,5)P_3$ has been characterized in a variety of systems. The physiological roles of various inositol polyphosphates and their cyclized derivatives have begun to appear. Enzyme purification and gene cloning have demonstrated the presence of multiple forms of phospholipase C and protein kinase C. However, the physiological meaning of this enzyme multiplicity is largely unknown.

Recent advances in the development of specific receptor ligands have greatly contributed to our understanding of the pharmacology of phospholipase C-coupled receptors, particularly in the CNS. In the case of serotoninergic receptors, both 5-HT_2 and 5-HT_{1C} subtypes are coupled to phospholipase C, while muscarinic cholinergic receptor subtypes M_1, M_2, and possibly M_3 are all linked to phosphoinositide metabolism. Recent success in the cloning of 5-HT_{1C} and various muscarinic receptor subtypes may provide a model for studying the relationship between receptor structures and the receptor coupling to phospholipase C. Mounting evidence supports the involvement of a G protein in coupling of the receptor to phospholipase C. However, despite intensive investigation, the identity of this G protein remains a mystery. It has become evident that both $InsP_3$ and DG arms of the phosphoinositide cycle activated by 5-HT_2 receptor stimulation participate in the smooth muscle contraction. Abnormalities in the level of α_1 -adrenergic receptors and this receptor-mediated phosphoinositide turnover have been found in animal models for some disease states, such as hypertension and seizure. In most, but not all, systems studied, there is little receptor reserve for α_1 -adrenergic and muscarinic cholinergic receptors coupled to phospholipase C. The glutamate receptor, especially the NMDA subtype, has been implicated in the process of learning and memory. These events may involve translocation of protein kinase C from the cytosol to the membrane following receptor stimulation. Supersensitivity of phosphoinositide breakdown mediated by α_1 -adrenergic, glutamatergic and 5-HT_{1C} receptors has been documented in numerous systems after denervation induced by various pharmacological manipulations. Conversely, evidence for both homologous and heterologous desensitization is available in a variety of cell-types following exposure to selective agonists for calcium-mobilizing receptors. Phosphorylation of the receptor protein or other regulatory components may be involved in

the process of desensitization; however, the exact role of protein kinase C activation in this regulatory event awaits further investigation. There is interregulation between receptors coupled to phospholipase C and other effector systems. Receptor-mediated phosphoinositide turnover can be either stimulated or inhibited by activation of other classes of cell-surface receptors. Future experimentation using broken-cell preparations is required to establish whether these observations reflect direct interactions between different classes of receptors. Emerging evidence also suggests that one of the targets of desensitization, receptor interregulation, stimulatory effects of sodium channel activators, and the inhibitory actions of therapeutic doses of lithium may be at the level of receptor-G protein-phospholipase C complex. Unraveling the nature of G protein coupled to phospholipase C is a major challenge for the years to come.

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