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MINISYMPOSIUM:

II. BIOCHEMICAL MECHANISMS IN THE PHOSPHATIDYLINOSITOL EFFECT

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Summary

Stimulated labeling of phospholipids from $^{32}P_i$ is a hallmark of activation of a variety of cell surface receptors. In the case of the central nervous system, the response can reflect muscarinic activation. Recent studies in nerve ending preparations indicate a postsynaptic site of action. Ca^{2+} is required for the expression of cholinergic stimulation of labeling in nerve ending preparations, but whether it plays a regulatory role is not yet known. While it is inferred that the receptor-ligand interaction leads to increased diacylglycerol availability, its source is not established. In experiments with muscarinic agents and ionophore added to nerve ending preparations, there is a potentiated loss of labeling from prelabeled polyphosphoinositides. It is suggested that phosphodiesteratic cleavage of polyphosphoinositides may be an early consequence of muscarinic receptor activation.

Phosphatidylinositol (PhI) is 1,2-diacyl-sn-glycero(3)-phospho(1)-D-myo-inositol (Fig. 1A). Much confusion over the nomenclature of the cyclitols and their lipids can be attributed to the previous use of mutually incompatible numbering systems (1). In recent history, the IUPAC Commission on Nomenclature renamed as D-1-myo-inositol 1-phosphate what had up until then been called the L-1-phosphate (2). The confusion was again compounded by a mix-up in the 1977 Commission report (3,4). The reader can at once be liberated of these past problems by examining Fig. 1B. It can be seen that the chair form of myo-inositol consists of one axial and five equatorial hydroxyls. If we imagine the molecule to be in the shape of a turtle with the axial hydroxyl (D-2) as its head, then the right front paw is D-1, the position phosphodiesterified to glycerol in inositol lipids. Higher inositides are additionally monoesterified with phosphate at D-4 (the left hind leg) or D-4 and D-5 (the tail), yielding phosphatidylinositol 4-phosphate (PhIP), or phosphatidylinositol 4,5-bisphosphate ($PhIP_2$), respectively. It is of interest from the structural standpoint that although there are nine possible stereoisomers of hexahydroxycyclohexane, the myo isomer is by far the most prevalent and the only cyclitol isomer known to occur in a natural phosphatide. The polyphosphoinositides, PhIP and $PhIP_2$, are present in cell membranes in much lower amounts than PhI. Much recent interest in PhI and its derivatives relate to the high proportion of arachidonate (20:4 ω 6 cis) in the 2 position of the glycerol moiety of the phosphoinositides, since arachidonate is a known precursor of physiologically important prostanoids (5).

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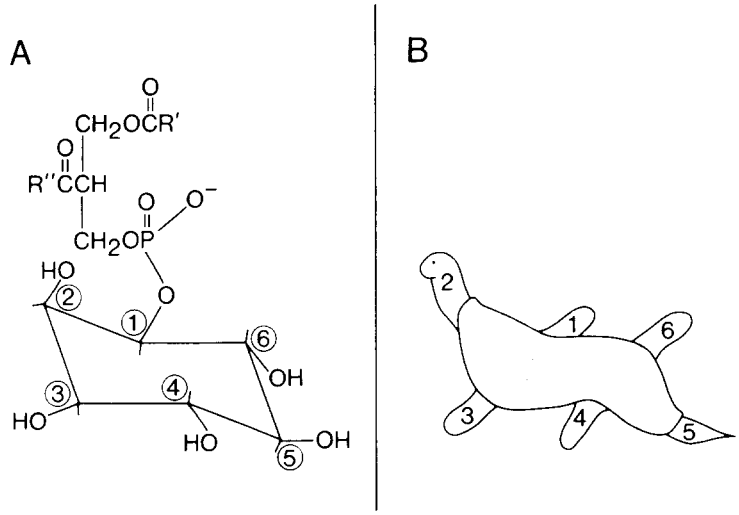


FIG. 1

A. Structure of phosphatidylinositol. The naturally occurring lipid is enriched in stearate in R' and arachidonate in R". B. The turtle's head is axial while its legs and tail are equatorial hydroxyls of myo-inositol in the prevalent chair form. The phosphatidyl group is attached at the D-1 (right forepaw) in all of the phosphoinositides. See text.

Stimulated lipid labeling. The stimulated labeling by $^{32}P_i$ of PhI and of phosphatidate (PhA) by incubation of various tissue preparations in the presence of ligands for which they have cell membrane receptors has been known for thirty years. For example, when carbamylcholine was added to pancreas slices, PhA and PhI showed increased incorporation of $^{32}P_i$ present in the incubation medium (6). The stimulation could be blocked by atropine, a known muscarinic cholinergic receptor antagonist. Our interest several years ago in the possible metabolic relationship between PhA and PhI led to the discovery that PhI is biosynthesized by the transfer of the phosphatidyl moiety from the liponucleotide CDP-diacylglycerol to myo-inositol, with the liberation of CMP (7,8). The liponucleotide was subsequently found to be formed from the enzymatic reaction of CTP with PhA (9,10). In de novo biosynthesis (Fig. 2), PhA is formed from fatty acyl CoA and glycerol phosphate or dihydroxyacetone phosphate (11). Labeled PhA can also form by the action of diacylglycerol kinase on ^{32}P-ATP and diacylglycerol, which arises from degradation of a number of preexisting lipids. Thus $^{32}P_i$ added to an incubation medium is converted intracellularly to ^{32}P-ATP and if diacylglycerol is available, labeled PhA and PhI will be formed. When incorporation of precursors of de novo lipid synthesis is measured under conditions in which $^{32}P_i$ incorporation into PhA and PhI is stimulated, a comparable stimulation of incorporation of label relative to the basal rate from glucose or glycerol is not seen (12). The results indicate that the stimulated incorporation of $^{32}P_i$ is more likely accounted for by phosphorylation of endogenously

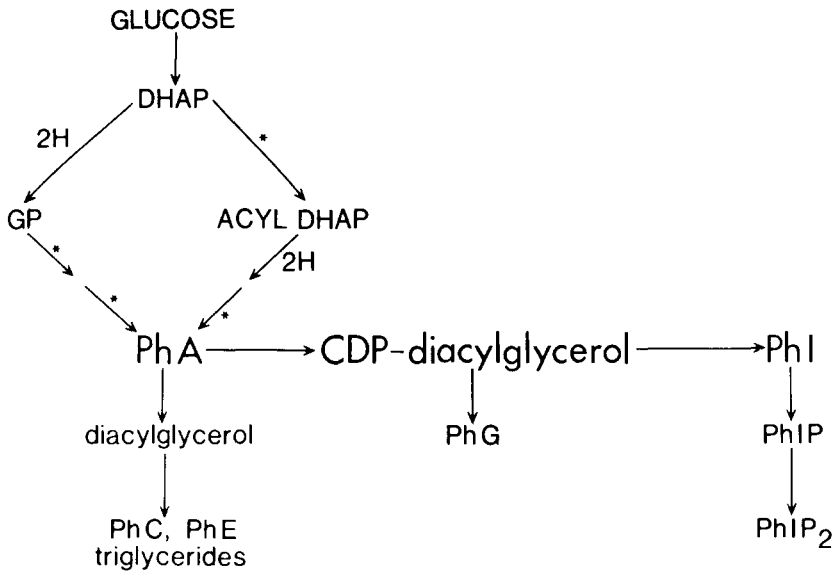
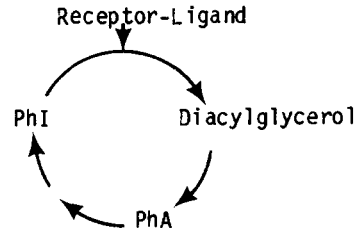


FIG. 2

Biosynthetic scheme of phospholipids illustrating precursor-product relationship of PhA and PhI. Dihydroxyacetone phosphate (DHAP) may be reduced to GP and be sequentially acylated to PhA, or alternatively, acylated in the 1 position, reduced to acyl DHAP and acylated to form PhA. Acylation steps are starred. CDP-diacylglycerol also serves as a precursor of the phosphatidylglycerols (PhG). PhA can be dephosphorylated to diacylglycerol, which then serves as precursor of phosphatidylethanolamine (PhE), phosphatidylcholine (PhC) and triglycerides.

generated diacylglycerol than by de novo biosynthesis of PhA and PhI. The production of diacylglycerol from preexisting lipid may then play a key role in stimulated labeling. A specific phospholipase that cleaves PhI to diacylglycerol and inositol monophosphate (a mixture of myo-inositol D-1 phosphate and D 1-2 cyclic phosphate) has been characterized (13). If the ligand-receptor interaction directly or indirectly stimulates this phosphodiesteratic cleavage, it can mediate a closed cycle as follows:



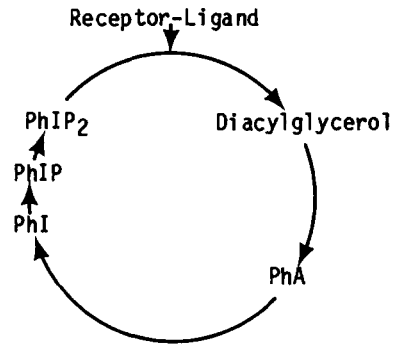
When an acidic extraction method is used in such labeling experiments, two additional labeled phospholipids are extracted: PhIP and $PhIP_2$. These polyphosphoinositides are highly labeled by $^{32}P_i$ in their monoester moieties, rather than in the phosphodiester, as a result of the actions of specific kinases and phosphatases that catalyze the reactions: PhI $\rightleftharpoons$ PhIP $\rightleftharpoons$ $PhIP_2$. As is discussed below, the polyphosphoinositides may play an important role in the mechanism of stimulated lipid labeling.

Studies in synaptosomes. The widespread occurrence among tissues of stimulated lipid labeling is well-documented (14). In addition to the pancreas, systems frequently employed to study this effect include the action of chemotactic peptide on leukocytes (15), of muscarinic agents on the avian salt gland (16), of vasopressin on hepatocytes (17), of thyrotropin on thyroid (18) and of thrombin on platelets (19). In general, the stimulated PhA-PhI labeling does not appear to involve cyclic nucleotide mechanisms or phospholipid methylation (20). In tissues in which the ligand-receptor interaction results in secretion, molar effects can be seen in the measured chemical amounts of phospholipids. For example, upon addition of ACh or pancreazymin to pancreatic acinar cells, PhI content is decreased and PhA increases (21). The secretory preparations suffer from the disadvantage that it is difficult to easily separate the quantitatively minor alterations in phospholipid metabolism attributable to signal transduction from those related to the subsequent secretory events. The various effector cell systems share the common property that they must be intact: once cells have been broken open, stimulated labeling can no longer be elicited. The ability of brain homogenates to retain an ACh-sensitive stimulated labeling effect can be attributed to the presence of nerve endings. These broken-off bits of neuronal terminals (synaptosomes) reseal into particles which may be considered anucleate neurons--they contain mitochondria, endoplasmic reticulum, and synaptic vesicles, etc. under conditions that maintain the vectorial properties of the plasma membrane. Studies in our laboratory have established that a "light" nerve ending fraction mediates the stimulated labeling (22).

In general, two types of experiments can be performed. In the first, one labels nerve ending preparations in an initial incubation and then examines increases or decreases in the prelabeled lipid following addition of ligands, blockers, etc. during a subsequent incubation period. In the second approach, $^{32}P_i$ can be added together with the various drugs to unlabeled preparations at the onset of the incubation. Using the latter paradigm, we found that the addition of 10^{-4} to 10^{-3}M ACh or carbamylcholine results in an atropine-sensitive increase in labeled PhA (about 100% stimulation) and PhI (about 70% stimulation) over a 30 minute period (22). PhIP and $PhIP_2$ are also highly labeled, but incorporation is not stimulated by muscarinic agonists. The stimulatory effects on PhA and PhI labeling are not accompanied by a change in the specific activity of intracellular ^{32}P-ATP, and thus represent increased turnover of the various phospholipids (23). If nerve endings are labeled in the presence or absence of ligand and then lysed and fractionated, the stimulated labeling can be shown to be localized to a plasma membrane-rich fraction (24). Ionophores mimic the cholinergic-stimulated labeling in nerve ending preparations, and their action can be blocked by the addition of EGTA. The combined presence of ionophore A23187 and carbamylcholine leads to a potentiation of the stimulation of PhA and PhI labeling (25), and a stimulated decrease in PhIP and $PhIP_2$ labeling is now seen (12). The addition of EGTA blocks the stimulation of lipid labeling by carbamylcholine, but the addition of atropine does not block the stimulatory effect of ionophore addition. In fact, when atropine is added to a medium containing both ionophore and carbamylcholine, the degree of stimulation reverts to that obtained with the addition of ionophore alone (12). The

results may be interpreted to indicate that Ca^{2+} mobilization is a direct consequence of the receptor-ligand interaction and precedes (and perhaps mediates) the stimulation of lipid labeling. While the findings are compatible with this interpretation, it should be cautioned that they indicate only that some basal Ca^{2+} level is necessary for the expression of the stimulated labeling, and do not necessarily indicate that Ca^{2+} is regulating the rate of lipid turnover. We have examined directly the possibility that Ca^{2+} fluxes are altered in nerve endings treated with muscarinic agents, and have found no evidence in support of this (L.A.A. Van Rooijen and B.W. Agranoff, in preparation).

The ionophore experiments in nerve endings indicate an interaction of the activated cholinergic receptor and Ca^{2+}, which may well be transmembrane in nature. More generally, a relationship between stimulated lipid labeling and Ca^{2+} has been inferred in all tissues that support stimulated lipid labeling, although its exact nature has yet to be defined. Since the cytosol is compartmented from mitochondrial and extracellular stores of Ca^{2+} whose concentration of Ca^{2+} are orders of magnitude greater, and since lipid labeling from $^{32}P_i$ can be evoked by ionophores that increase intracellular Ca^{2+}, it is tempting to speculate that the receptor-ligand interaction initiates a cascade which elevates cytosolic calcium. PhA itself has been shown to be a Ca^{2+} ionophore (26). Since the production of diacylglycerol may be Ca^{2+}-dependent, its release and resynthesis to PhA could be part of an amplification mechanism whereby large amounts of Ca^{2+} are mobilized. The demonstration of a protein kinase that is activated by diacylglycerol (27) also suggests the participation of a regulatory process. Of further possible relevance is the B-50 protein whose phosphorylation is believed to regulate the activity of PhIP kinase (28). Polyphosphoinositides are known to bind Ca^{2+} tightly, hence their metabolism could lead to altered local Ca^{2+} availability in the region of the plasma membrane. A breakdown of polyphosphoinositides was observed upon muscarinic stimulation of iris muscle (29) and has also been seen in synaptosomes (30), particularly combined in the presence of ionophore plus muscarinic agonist (12). More recently, the specific breakdown of $PhIP_2$ has been reported in stimulated parotid gland (31). This suggests that the labeling cycle may be extended as follows:



In the case of nerve endings, there is no known secretory or contractile process associated with the addition of muscarinic agents and no clear indication of the functional significance of the stimulated labeling. Interest in the effect is however high, since the phenomenon represents a rare biochemical "handle" of the activation of a CNS muscarinic receptor. The potential relevance of the phenomenon to brain function is exemplified by the recent demonstration of selective loss of cholinergic fibers in the cortex in Alzheimer's disease (32).

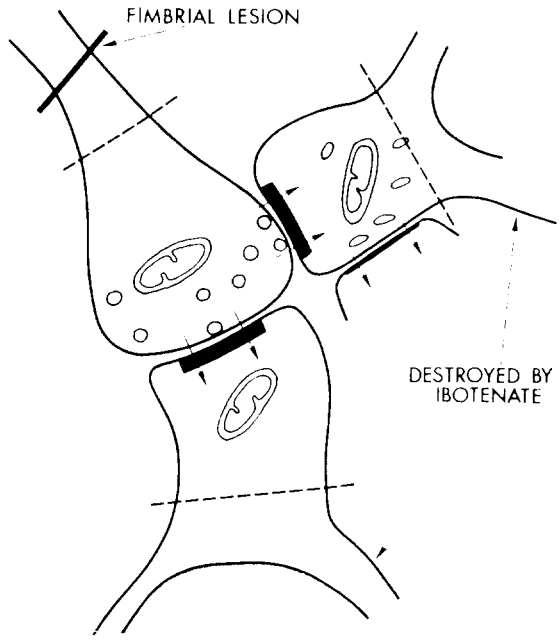


FIG. 3

Diagram of synaptic arrangements in brain that could give rise to nerve endings containing integral postsynaptic membranes. See text.

We have pursued the question of whether the effect is pre- or postsynaptic. The "typical" synaptosome, as we might encounter it in a textbook, is a round bag containing mitochondria and synaptic vesicles and may have an attached synaptic complex, including a bit of postsynaptic membrane of its former neighboring neuron. Since $^{32}P_i$ is converted to $\gamma\,^{32}P$-ATP inside the resealed nerve ending, one may be led to conclude that the stimulated labeling is presynaptic: $^{32}P_i$ enters the nerve ending, is converted to $\gamma^{32}P$-ATP and then enters lipids via cytosolic or membrane-bound diacylglycerol kinase or PhI/PhIP kinases. By analogy with experiments in non-brain preparations in which cholinergic stimulation leads to secretion, however, one would expect the effect to be postsynaptic. In order to reconcile these opposing interpretations, we examined the two possibilities by means of hippocampal lesions in the guinea pig brain. Fimbrial lesions block the major cholinergic input to the hippocampus, but are presumed to leave intact the postsynaptic cholinoceptive surfaces (33). Intracerebral injections of the excitatory toxin ibotenate, on the other hand, should destroy intrinsic neurons (and their postsynaptic receptor surfaces) and leave the cholinergic presynaptic terminals from the septal nuclei intact (34). The validity of these assumptions was tested with known markers for the pre- and postsynaptic terminals: cholineacetyltransferase activity and quinuclidinyl benzilate (QNB) binding, respectively. The stimulated labeling in fact corresponded most closely to a postsynaptic distribution (33,34). How then is this result reconciled with the expectation that stimulated labeling be presynaptic, based on the ultrastructural appearance of synaptosomes? We believe that the nerve

ending particles that mediate the effect contain postsynaptic membranes as an integral part of the plasma membrane. This will be the case if there is a presynaptic input to the nerve terminal, i.e., that it is cholinoceptive in addition to itself being presynaptic to a third neuron (see Fig. 3). The nerve ending particle that mediates stimulated labeling could alternatively be a pinched-off dendrite, or "dendrosome" (35).

In still other studies, the pharmacological nature of the CNS muscarinic receptor has been studied by comparison of QNB-binding and percent stimulation of PhI and PhA labeling (Fisher, Klinger and Agranoff, in preparation). Muscarinic agents can be classified as strong and weak agonists in regard to their ability to stimulate lipid labeling. While they all displace QNB, the weak agonists have some antagonist activity. High and low affinity receptors can be identified, and the stimulated lipid labeling is found to correlate with occupation of the low affinity sites.

Whether the detailed operation of the stimulated lipid labeling in such diverse tissues as nerve cells and platelets subserve the same or even similar purposes remains to be elucidated. For the present, one is more impressed by commonality than by differences, so that what is learned in one system is likely to have significance for another.

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