length about 3 cm), for example, the range of roughness sensitivity can probably be expanded to 0.3 to 3 cm, thus improving the discrimination capability (*18, 20, 25*). The development of a multispectral spaceborne radar system is expected to take place in the next few years.

Orbital radar will also allow us to develop a data base for the interpretation of images to be obtained from planetary missions. For Venus, and possibly Titan, radar will be the only means of mapping the planet's surface through the continuous and complete cloud cover.

Because of the complex nature of SAR's on orbital platforms, the successful development of the Seasat SAR was a key technical advancement. Major technological developments are still needed before multispectral orbiting SAR sensors can become operational, particularly in the area of digital, real-time processing. Because the radar sensor basically provides a Doppler time-delay history of each point target, thousands of computational operations are required to generate a single image element. This processing requirement, combined with the desire to have large swath mapping with high resolution, requires extremely fast processing hardware which is just at the limit of present-day technology.

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26. This article represents the results of one phase of research carried out at the Jet Propulsion Laboratory, California Institute of Technology, under contract NAS7-100, sponsored by the Non-Renewable Resources Office, Office of Space and Terrestrial Applications, NASA. I thank M. Abrams, R. Blom, M. L. Bryan, M. Daily, T. Dixon, J. Ford, R. S. Saunders, and H. Stewart from Jet Propulsion Laboratory for their helpful discussions.

Phospholipid Methylation and Biological Signal Transmission

Fusao Hirata and Julius Axelrod

The mechanisms by which specific biochemical signals are transmitted through membranes is a problem of major importance in biology. Biochemical messages in the form of neurotransmitter, peptide hormone, lectin, and immunoglobulin ligands are recognized by and bind to specific receptor macromolecules on the outer surface of cell membranes. These interactions then initiate chemical and physical changes in membranes which in turn allow cells to carry out their specific function. Experiments in our laboratory in the past 3 years have shown that enzymatic methylation of phospholipids play an important role in the transduction of receptor-mediated signals through the membranes of a variety of cells.

Phospholipid Methylation, Translocation, and Membrane Fluidity

We observed that the addition of Mg^{2+} ions to adrenal medulla homogenates stimulated the incorporation of the radioactive methyl group of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (SAM), a methyl donor, into a lipid fraction (*1*). This prompted us to examine the identity of the [^{3}H]methylated lipids, which we separated by extraction with organic solvents and by thin-layer chromatography. We found that the [^{3}H]methyl groups were incorporated into phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*,-dimethylethanolamine, and phosphatidylcholine. After further work, we found two enzymes in the adrenal medulla that converted phosphatidylethanolamine to phosphatidylcholine by successive methylations with SAM (*1*). Synthesis of phosphatidylcholine by two methyltransferases had been proposed previously (*2*) in genetic variants of the yeast *Neurospora crassa*.

The two phospholipid methyltransferases in the adrenal medulla have different properties (Fig. 1). The first enzyme (methyltransferase 1) converts phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine, requires

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0036-8075/80/0905-1082$02.00/0

Summary. Many types of cells methylate phospholipids using two methyltransferase enzymes that are asymmetrically distributed in membranes. As the phospholipids are successively methylated, they are translocated from the inside to the outside of the membrane. When catecholamine neurotransmitters, lectins, immunoglobulins or chemotaxic peptides bind to the cell surface, they stimulate the methyltransferase enzymes and reduce membrane viscosity. The methylation of phospholipids is coupled to Ca^{2+} influx and the release of arachidonic acid, lysophosphatidylcholine, and prostaglandins. These closely associated biochemical changes facilitate the transmission of many signals through membranes, resulting in the generation of adenosine 3′,5′-monophosphate in many cell types, release of histamine in mast cells and basophils, mitogenesis in lymphocytes, and chemotaxis in neutrophils.

Mg^{2+}, has an optimal *p*H about 7.0 and a low K_m (Michaelis constant, about 2 μM) for SAM. The second enzyme (methyltransferase II) catalyzes the stepwise methylation of phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine. This enzyme, which has been described previously [see (*3*)], does not require Mg^{2+}, has a high K_m (about 100 μM) for SAM, and an optimal *p*H of 10.0. The two methyltransferases are localized in the microsomes and mitochondria of the adrenal medulla. They have been found in all tissues thus far examined: brain (*4*), red cells (*5*), lymphocytes (*6*), mast cells (*7*), and basophils and neutrophils (*8*).

The molecular organization of membranes consists of a lipid bilayer in which biochemically active proteins are embedded. The lipid bilayer of biological membranes is mainly composed of phospholipids and provides a fluid matrix for protein organization and movement (*9*). Lipids are asymmetrically distributed in plasma membranes with phosphatidylethanolamine largely facing the cytoplasmic side and phosphatidylcholine mainly orientated toward the outside (*10*). An important question for understanding membrane function is how lipid asymmetry comes about. The asymmetrical distribution of phosphatidylethanolamine, the substrate for methyltransferase I, and phosphatidylcholine, the product of methyltransferase II, led to a study of the localization of these enzymes in membranes (*5*). For these experiments, erythrocyte ghosts were used, since they can be prepared right-side-out and inside-out (*11*).

Rat erythrocyte ghosts were first examined for the presence of the two phospholipid methyltransferase enzymes. Both enzymes were found to be present in the erythrocyte membranes (*5*). The topology of the lipid methyltransferases was studied by introducing [*methyl*-^{3}H]SAM into right-side-out erythrocyte ghosts by incubation at 4°C overnight. After the methyl donor was introduced into the cells the enzyme reaction was started by incubation at 37°C. This led to the incorporation of the [^{3}H]methyl group into phospholipids. Incubating right-side-out erythrocyte ghosts with the radioactive methyl donor present on the outside of the membrane resulted in negligible methylation of phospholipids. When the membranes were turned inside-out so that the cytoplasmic side faced out, there was an increase in the methylation of phospholipids upon the addition of SAM. After introduction of SAM into the interior of an inside-out membrane, there was little methylation of phospholipids. It was concluded that methylation of phospholipids begins on the cytoplasmic side of the membrane, where the substrate phosphatidylethanolamine for methyltransferase I is localized.

The asymmetric localization of the phospholipid methylating enzymes was demonstrated by selective proteolytic digestion with trypsin (*5*). When added to cells, trypsin will only digest proteins that face the outside of the membrane. When right-side-out erythrocytes were incubated with trypsin, methyltransferase II, the enzyme that synthesizes phosphatidylcholine, was destroyed. Conversely, when inside-out membranes were treated with trypsin, methyltransferase I was digested and methyltransferase II was spared. These experiments showed that methyltransferase I faces the cytoplasmic side of the membrane together with its substrate, while methyltransferase II, which successively methylates phosphatidyl-*N*-monomethylethanolamine to phosphatidylcholine, faces the outside of the membrane.

The question of whether phospholipids are translocated across the membrane during the methylation process was examined by treating erythrocyte ghosts with phospholipase C (E.C. 3.1.4.3) (*12*). This enzyme removes the polar head of phospholipids facing the outside surface of the membrane together with the enzymatically attached radioactive methyl group. By using phospholipase C and inside-out and right-side-out erythrocyte ghosts to which [*methyl*-^{3}H]SAM was added, it was found that methylated phospholipids are translocated from the cytoplasmic side of the membrane to the exterior surface. The enzymatically facilitated flip-flop of the methylated phospholipids occurred rapidly, in less than 2 minutes. This is different from the much slower translocation of phospholipids in liposomes (*13*). A rapid translocation of phosphatidylethanolamine has also been reported in membranes of *Bacillus megatherium* (*14*).

The methylation and the rapid vectoral rearrangement of phospholipids had an influence on membrane fluidity. The effect of methylation and translocation of phospholipids on membrane viscosity was examined by treating erythrocyte ghosts with 1,6-diphenyl-1,3,5-hexatriene (DPH). The anisoscopy of DPH fluorescence by polarized light reflects membrane viscosity (*15*). When phospholipids were methylated by introducing SAM into resealed erythrocyte ghosts, there was a decrease in the microviscosity of the membrane as measured by DPH (*16*). The decreased microviscosity was abolished when methylation of phospholipids was inhibited by *S*-adenosylhomocysteine (SAH). When varying concentrations of SAM were used, the synthesis of phosphatidyl-*N*-monomethylethanolamine had an important effect on membrane fluidity. Further methylation to phosphatidylcholine had little influence on membrane viscosity. Phosphatidyl-*N*-monomethylethanolamine was found to be deeply embedded in the membrane as measured by treatment with phospholipase C (*5*). Thus, the methylation of phosphatidylethanolamine or the rapid transit of the monomethylated lipid, or both, appear to influence membrane fluidity.

β-Adrenergic Receptors Regulate Phospholipid Methylation

The discovery of two methyltransferases and their ability to translocate phospholipids suggested that they might have important biological functions. Immature red cells (reticulocytes) have β-adrenergic receptors that are coupled to adenylate cyclase (E.C. 4.6.1.1) (*17*). These cells can be prepared as nonleaky ghosts. This made it possible to introduce [*methyl*-^{3}H]SAM inside the reticulocyte ghost and to examine the effect of

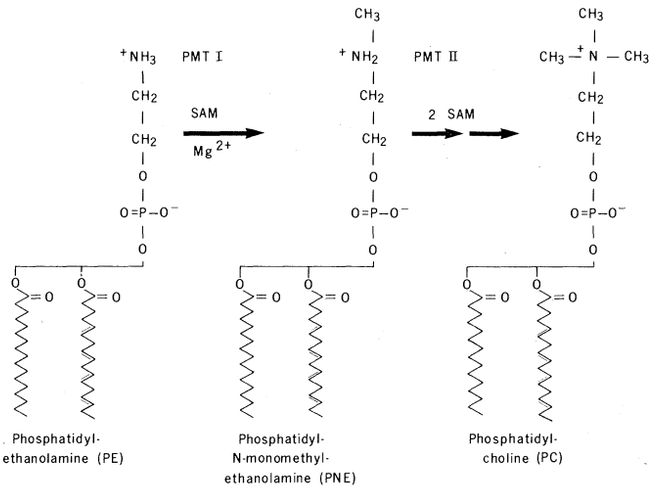

Fig. 1. The enzymatic conversion of phosphatidylethanolamine to phosphatidylcholine. Abbreviations: *PMT I*, phospholipid methyltransferase I; *PMT II*, phospholipid methyltransferase II; *SAM*, *S*-adenosyl-L-methionine.

stimulating the β-adrenergic receptor with catecholamine agonists on phospholipid methylation, membrane fluidity, and adenylate cyclase (*18*).

Rats were stimulated to form reticulocytes by the injection of phenylhydrazine. The [*methyl*-^{3}H]SAM was introduced into preparations of reticulocyte ghosts and the effect of a potent β-adrenergic agonist, L-isoproterenol, on the incorporation of [^{3}H]methyl group into phospholipids, was examined (*18*). L-Isoproterenol caused an increase in the incorporation of [^{3}H]methyl group into phospholipids in a dose-dependent manner. The increased methylation of phospholipids by L-isoproterenol did not occur in leaky reticulocyte ghosts, indicating that the structural integrity of the membrane was necessary. By using phospholipase C it was found that stimulation with isoproterenol caused a flip-flop of the methylated phospholipids from the cytoplasmic side to the outer surface of the reticulocyte membrane. The stimulation of phospholipid methylation by isoproterenol was stereospecific. The order of potency for agonists was L-isoproterenol > L-adrenaline > L-noradrenaline. Propranolol, a β-adrenergic antagonist, blocked the isoproterenol-stimulated methylation while phentolamine, an α-adrenergic antagonist, had no effect (*17*). The value for half-maximal activation of phospholipid methylation by isoproterenol was close to that necessary for half-maximal activation of adenylate cyclase in rat reticulocyte ghosts. Previous work had shown that β-adrenergic receptor activity is sensitized by guanosine triphosphate (GTP) (*19*). Consistent with this was the observation that GTP increased the ability of isoproterenol to stimulate phospholipid methylation. In the presence of the guanyl nucleotide the K_a value (activation constant) for phospholipid methylation by isoproterenol was reduced from 30 μ*M* to 0.8 μ*M*, but the V_{max} (maximum velocity) remained unchanged. All of these observations indicated that binding of the β-adrenergic receptor on the cell surface by catecholamines activated the methylation of phospholipids in reticulocyte membranes (Fig. 2).

When the β-adrenergic receptor binds to isoproterenol, it couples with the adenylate cyclase to generate adenosine 3′,5′-monophosphate (cyclic AMP) (Fig. 2). Increased phospholipid methylation could be caused by the interaction of the β-adrenergic receptor with its ligand or by the direct activation of adenylate cyclase. Cholera toxin and sodium fluoride can stimulate adenylate cyclase directly, bypassing the receptor. The addition of either of these compounds had little effect on phospholipid methylation (*18*). Thus, stimulation of membrane lipid methylation is due to the binding of the β-adrenergic receptor to its agonists and is not secondary to the activation of adenylate cyclase.

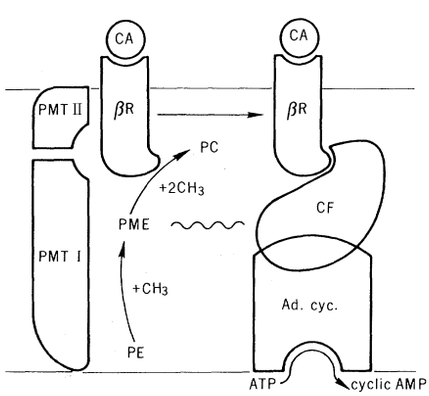

Fig. 2. Phospholipid methylation and β-adrenergic receptor coupling. When catecholamine (*CA*) binds to β-adrenergic receptor (*βR*), it stimulates phospholipid methyltransferase I (*PMT I*) and phospholipid methyltransferase II (*PMT II*). This increases the methylation of phosphatidylethanolamine (*PE*) to phosphatidyl-*N*-monomethylethanolamine (*PME*) and to phosphatidylcholine (*PC*). As the phospholipids are methylated they flip-flop and increase fluidity (~). This facilitates the lateral mobility of the β-adrenergic receptor to interact with the guanylnucleotide coupling factor (*CF*) and adenylate cyclase (*Ad. cyc.*) to generate cyclic AMP.

Phospholipid methylation increases the fluidity of reticulocyte membranes (*18*) and this should enhance the lateral mobility and rotation of the β-adrenergic receptor. If this were so, then increased phospholipid methylation should facilitate the coupling of the receptor on the outer surface with adenylate cyclase facing the cytoplasmic side of the membrane. Increasing the methylation of lipids by introducing SAM into the reticulocyte ghosts increased the isoproterenol-stimulated adenylate cyclase more than twofold (*18*). The greatest change in coupling of the receptor with the cyclase enzyme occurred at a concentration of SAM which caused activation of methyltransferase I and the generation of phosphatidyl-*N*-monomethylethanolamine. Increasing membrane fluidity by the addition of vaccenic acid to turkey erythrocytes also facilitated receptor adenylate cyclase coupling, whereas decreasing fluidity with cholesterol reduced hormone receptor cylase coupling (*20*). From these experiments it appears that the uncoupled β-adrenergic receptor depresses methyltransferase activity. This is compatible with the observation that solubilization of the membranes with nonionic detergents, a procedure that would separate the enzyme and receptor, increases methylation activity. With the interaction of the β-receptor and an agonist, the suppression of the enzyme might be overcome and phospholipid methylation increased. More monomethylphospholipids would be generated which would decrease membrane viscosity. This would allow for a greater lateral movement of the receptors and a greater chance to couple with adenylate cyclase (Fig. 2).

HeLa cells contain β-adrenergic receptors that can couple with adenylate cyclase. This property gave us an opportunity to study the role of the β-adrenergic receptor in the activation of phospholipid methylation in another cell line. Since SAM does not easily penetrate intact HeLa cells, phospholipid methylation was measured by the incorporation of the [^{3}H]methyl group into the lipid fraction from [^{3}H]methionine. SAM is readily synthesized from [^{3}H]methionine in these cells. Phospholipid methylation was stimulated by catecholamines with an order of potency characteristic of a β-adrenergic receptor: isoproterenol

> adrenaline > noradrenaline (*21*). There was a close parallelism between the dose response curves of the various catecholamines for phospholipid methylation and for the formation of cyclic AMP. Both phospholipid methylation and cyclic AMP formation were blocked by the β-adrenergic antagonist, propranolol, and not by the α-adrenergic antagonist, phentolamine.

Phospholipid Methylation Regulates β-Adrenergic Receptors

The cellular response to many neurotransmitters and hormones is regulated by changes in receptor number and coupling of membrane-bound enzymes, a process that generates intracellular effectors. β-Adrenergic receptor numbers can be measured by using a radioactive ligand, such as [^{3}H]dihydroalprenolol (*22*), which has a high affinity for the receptors. The effect of phospholipid methylation on β-adrenergic receptor numbers was examined in rat reticulocyte ghosts. The methylation of phospholipids was stimulated by incubating rat reticulocyte ghosts at 37°C with SAM for about 60 minutes (*23*). Under this condition, the number of β-adrenergic binding sites increased about 30 to 40 percent. When the methyltransferase inhibitor, SAH, was introduced into the ghosts together with the methyl donor, both phospholipid methylation and the appearance of new β-adrenergic receptors were inhibited. The increase in the number of β-adrenergic sites due to methylation was temperature-dependent, showed no change on incubation at 4°C, and caused no change in the affinity of [^{3}H]dihydroalprenolol. Incubating reticulocyte ghosts with varying concentrations of SAM indicated that the number of β-adrenergic binding sites increased with the synthesis of phosphatidylcholine but not with the synthesis of phosphatidyl-*N*-monomethylethanolamine (*23*). The increased number of receptor sites did not require new protein synthesis. From these studies it appears that receptors are hidden in membranes and become available to specific ligands when the synthesis of phosphatidylcholine is increased. Increased phospholipid methylation also enhances the binding of ^{125}I-labeled human growth hormone in membranes from mammary glands of lactating mice (*24*).

In another experiment with HeLa cells, it was found that the number of β-adrenergic receptors can rapidly change depending on the extent of phospholipid methylation. 3-Deazaadenosine (3-DZA) can enter cells and then form metabolites that inhibit transmethylation reactions. These methyltransferase inhibitors are useful for examining the physiological role of transmethylation reactions. When 3-DZA was added to HeLa cells, the number of β-adrenergic receptors and the formation of cyclic AMP were rapidly reduced (*25*). In the range of concentrations of 3-DZA used, phospholipid methylation was inhibited to a much greater extent than protein or nucleotide methylation. When the methyltransferase inhibitors were removed from the cells by washing, the β-adrenergic receptor number returned to normal within 1 hour. Thus, the degree of phospholipid methylation can mask or unmask hidden receptors.

Cells exposed to reduced concentrations of hormones or transmitters rapidly adapt by becoming supersensitive. In contrast, when cells are exposed to excessive amounts of hormones or transmitters they become desensitized (*26*). As will be described below, phospholipid methylation is closely coupled with phospholipase A_2 (E.C. 3.1.1.4) activation, and phospholipase A_2 appears to be involved in the desensitization of β-adrenergic receptors. Cells of the C_6 rat glioma astrocytoma line have β-adrenergic receptors that are coupled to adenylate cyclase. These cells can be rapidly desensitized after repeated exposure to β-adrenergic agonists, such as isoproterenol (*27*). When C_6 astrocytoma cells were treated with phospholipase A_2 inhibitors, such as mepacrine or tetracaine, refractoriness to cyclic AMP formation after successive treatments with isoproterenol was abolished (*28*). When cells were treated with phospholipase A_2 activators, such as phorbol esters or mellitin, the astrocytoma cells were rapidly desensitized. This desensitization is probably mediated by one or more products of phospholipase A_2 activity, such as lysophosphatidylcholine or arachidonic acid or its prostanglandin metabolites.

In addition to having β-adrenergic receptors, cultured C_6 astrocytoma cells have benzodiazepine receptors (*29*). Benzodiazepines are a group of drugs that have antianxiety properties. The presence of more than one type of receptor in these cells provided an opportunity to examine whether the occupation of both types of receptor can effect methyltransferase in an additive manner (*30*). C_6 astrocytoma cells were incubated with [*methyl*-^{3}H]methionine and β-adrenergic agonists were then added. The β-adrenergic agonists stimulated the incorporation of [^{3}H]methyl groups into phospholipids in a dose-dependent manner. The ability of the agonists to increase methylation was closely correlated to their capacity to stimulate adenylate cyclase (*30*).

Benzodiazepine also stimulated the incorporation of [^{3}H]methyl groups into phospholipids of C_6 astrocytoma cells in a dose-dependent manner (*30*). The potency of several benzodiazepine-like drugs matched their potency in displacing the agonist [^{3}H]diazepam from the receptor binding sites. Benzodiazepine and β-adrenergic agonists could act on the same pool of methyltransferase enzymes or on separate domains associated with the specific receptors. If the benzodiazepine and β-adrenergic agonists increased phospholipid methylation in the same methyltransferase membrane pool, then stimulation of both receptors would elevate methylation no more than if either receptor received maximum stimulation. If methyltransferases were associated with a specific receptor on different domains in the membrane the effect on phospholipid methylation with the simultaneous application of both types of agonists would be additive. When β-adrenergic and benzodiazepine agonists were added together at a maximum concentration, phospholipid methylation was increased in an additive manner (*30*). These observations indicate that different receptors are located in separate areas in the membrane and are associated with their own complement of methyltransferase enzymes. Thus, activation of each receptor would affect the phospholipid associated changes in viscosity only in the vicinity of that receptor. This is consistent with the finding that stimulation of fibroblasts with the lectin concanavalin A (Con A) results in restricted lateral mobility of receptors (*31*).

Phospholipid Methylation, Ca^{2+} Influx, and Histamine Release

For examining the effects of phospholipid methylation on the transmission of other biological signals, rat mast cells were used. Mast cells have specific receptors for immunoglobulin E (IgE) (*32*). The bridging of cell-surface IgE molecules by either multivalent antigens or divalent antibodies to IgE triggers the release of histamine from these cells (*33*). For examining the relation between phospholipid methylation and histamine release, Con A was initially used. Con A provokes clustering of IgE and sets off a series of intracellular events that lead to secretion of histamine (*32*). Mast

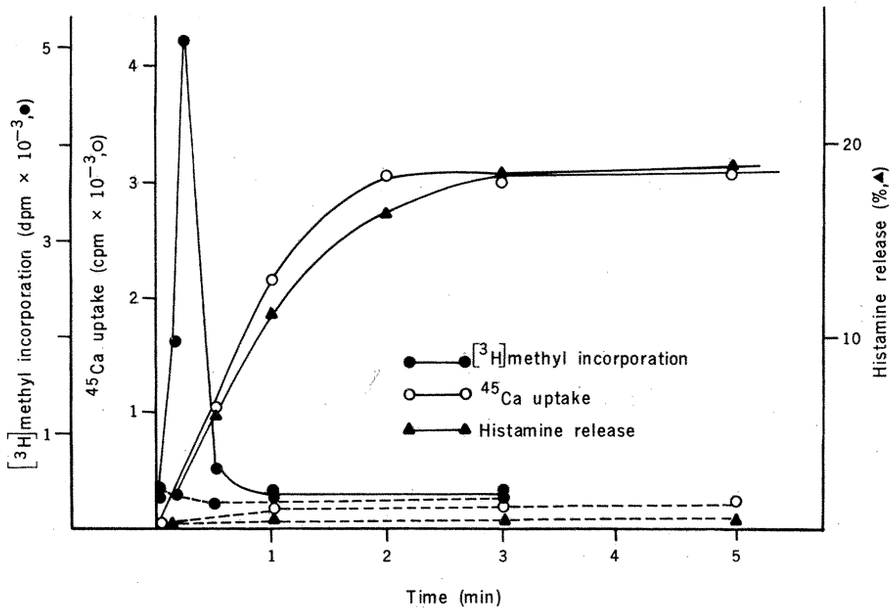

Fig. 3. Phospholipid methylation, Ca^{2+} influx, and histamine release in rat mast cells. Mast cells were first incubated with [^{3}H]methionine or $^{45}Ca^{2+}$. Incorporation of [^{3}H]methyl groups into phospholipids, $^{45}Ca^{2+}$ influx, and histamine release were measured after various treatments. Cells were treated with either divalent $F(ab')_2$ (——) or monovalent Fab' (---) fragments of antibodies to RBL cells. [From (*40*)]

cells were obtained from rat peritoneal fluid and incubated with L-[*methyl*-^{3}H]methionine. Methionine is rapidly converted to SAM in these cells. Treating the cells with Con A resulted in a rapid incorporation of the [^{3}H]methyl group into the lipid fraction followed by a release of histamine (*7*). After about 3 minutes there was a decrease in the methylated phospholipids, suggesting further metabolism of the lipids. In the absence of Con A, there was little lipid methylation or histamine release. When the binding of Con A was blocked with β-methylmannoside or the temperature lowered to 4°C, the Con A–mediated histamine release and the increased methylation were abolished (*7*).

In additional experiments to show the association between Con A–stimulated phospholipid methylation and histamine release, the methyltransferase inhibitor 5′-deoxyisobutylthio-3-deazaadenosine (3-deaza-SIBA) or 3-DZA together with homocysteine-thiolactone were used. 3-Deaza-SIBA acts directly as a methyltransferase inhibitor (*34*); 3-DZA is metabolized intracellularly to an analog of SAH and also inhibits the metabolism of SAH (*34*). Incorporation of these compounds into mast cells reduced the increase in phospholipid methylation as well as in histamine release after treatment with Con A (*7*).

Mast cells are deficient in phosphatidylserine, a precursor of phosphatidylethanolamine (*35*). The addition of phosphatidylserine to rat mast cells increased the release of histamine after treatment with Con A. When phosphatidylserine was omitted there was also a reduction in the incorporation of [^{3}H]methyl group into phospholipids after treatment with Con A (*7*). Upon the addition of [^{14}C]-phosphatidylserine to mast cells, Con A stimulated the incorporation of radioactivity into mast cells. An examination of the products showed the presence of radioactive mono-, di-, and trimethylated phospholipids as well as lysophosphatidylcholine (*7*). All of these experiments indicate that the binding of Con A triggers a cascade of biochemical reactions in mast cell membranes as follows: phosphatidylserine $\xrightarrow{-CO_2}$ phosphatidylethanolamine $\xrightarrow{+CH_3}$ phosphatidyl-*N*-monomethylethanolamine $\xrightarrow{+2\ CH_3}$ phosphatidylcholine $\xrightarrow{-\text{fatty acid}}$ lysophosphatidylcholine. In the course of these reactions, the substrates phosphatidylserine and phosphatidylethanolamine, which are usually localized on the cytoplasmic side of the membrames (*10*), are probably translocated to the outer surface. During the transit and methylation of phospholipids through the membrane a reduction of viscosity might occur, as has been shown with erythrocyte membranes (*16*). An increase in fluidity should facilitate lateral mobility and clustering of cell surface receptors.

Histamine is released from mast cells by exocytosis involving fusion of the biogenic amine–containing vesicles with the plasma membrane (*36*). The presence of Ca^{2+} is necessary for the exocytotic release of histamine. Phospholipid methylation appears to be involved in the Ca^{2+}-evoked histamine release. The presence of Ca^{2+} is also required for the activation of phospholipase A_2, the enzyme that transforms phosphatidylcholine to lysophosphatidylcholine (*37*). Lysophosphatidylcholine has been shown to be detergent-like and a fusogen; it also stimulates mast cell secretion (*38*). Inhibition of phospholipase A_2 with mepacrine (quinacrine) reduces the release of histamine (*7*).

The availability of antibodies against IgE and its receptors made it possible to examine the relations between phospholipid methylation, bridging of cell surface IgE receptors, Ca^{2+} influx, and the release of chemical mediators from mast cells. It has been demonstrated that antibodies against IgE receptors or their $F(ab')_2$ fragments cause an influx of $^{45}Ca^{2+}$ into mast cells (*39*). This is followed by release of histamine. The effect of antibodies to the IgE receptors of rat basophilic leukemia (RBL) cells on phospholipid methylation in mast cells was examined (*40*). The $F(ab')_2$ fragments of antibodies to RBL cells induced a rapid incorporation of [^{3}H]methyl groups into lipids. Maximum phospholipid methylation was reached very quickly, within 15 seconds, and had declined to base line by 30 seconds (Fig. 3). The uptake of $^{45}Ca^{2+}$ and release of histamine were also measured after stimulation with $F(ab')_2$ fragments of antibodies to RBL cells (*40*). The uptake of $^{45}Ca^{2+}$ followed the rise and fall of phospholipid methylation and reached a plateau in about 2 minutes. Maximum histamine release was achieved within 2 to 3 minutes. When mast cells were stimulated with a Fab′ monomer fragment of antibodies to RBL cells there was no increase in phospholipid methylation (Fig. 3). Monovalent fragments also failed to induce $^{45}Ca^{2+}$ uptake or histamine release (Fig. 3). Stimulation of mast cells with antibodies to IgE after the receptors were saturated with monoclonal IgE increased the incorporation of [^{3}H]methyl group into lipids, $^{45}Ca^{2+}$ influx, and histamine release in a similar time sequence as that found after stimulating unsensitized mast cells with $F(ab')_2$ fragments of antibodies to RBL cells (*40*). All of these findings demonstrated that bridging of IgE receptors is necessary to initiate phospholipid methylation, $^{45}Ca^{2+}$ influx, and histamine release in mast cells (Fig. 4).

The observation that phospholipid methylation precedes $^{45}Ca^{2+}$ influx and

histamine release suggested that phospholipid methylation might be a prerequisite for Ca^{2+} influx into mast cells. To test this hypothesis, the effect of the methyltransferase inhibitor 3-deaza-SIBA (*34*) on the incorporation of [^{3}H]-methyl groups into lipid and $^{45}Ca^{2+}$ influx was examined. Prior incubation of the mast cells with 3-deaza-SIBA inhibited phospholipid methylation by 90 percent, $^{45}Ca^{2+}$ influx by 85 percent, and histamine release by 78 percent after the cells were challenged with antibodies to RBL cells (*40*). By using varying concentrations of the inhibitors all three reactions were reduced in a dose-dependent manner. Similar results were obtained with another methyltransferase inhibitor, 3-DZA. It is apparent from these experiments that phospholipid methylation influences Ca^{2+} influx and the subsequent release of histamine. How phospholipid methylation affects Ca^{2+} influx in membranes is not yet known. In the course of phospholipid methylation, membrane viscosity is likely to be reduced and the resultant increase in fluidity would affect the microenvironment in which IgE receptors are located. This, in turn, may open calcium ion channels and activate the Ca^{2+} requiring phospholipase A_2 (Fig. 4).

Phospholipid methylation also affects the efflux of Ca^{2+}. The efflux of Ca^{2+} from cells is regulated by Ca^{2+}-dependent adenosinetriphosphatase (Ca^{2+}-ATPase). This enzyme is surrounded by an annulus of phospholipids, which is necessary for it to function (*41*). The effect of phospholipid methylation on Ca^{2+}-ATPase and Ca^{2+} efflux was examined in rat erythrocytes (*42*). Human erythrocyte membranes were first incubated with SAM and $MgCl_2$, and then Ca^{2+}-ATPase activity was determined. After the introduction of SAM into erythrocyte ghosts, phospholipid methylation was increased and so was Ca^{2+}-ATPase. The enhancement of Ca^{2+}-ATPase with the methyl donor was inhibited by the methyltransferase inhibitor, SAH. The maximum increase in ATPase activity was achieved at a concentration of SAM that stimulated methyltransferase I and the synthesis of phosphatidyl-*N*-monomethylethanolamine (*4*). Since methyltransferase I is mainly responsible for decreasing membrane viscosity (*16*), these findings suggest that changes in fluidity affect Ca^{2+}-ATPase activity. Consistent with this interpretation is the observation that Ca^{2+}-ATPase activity in sacroplasmic reticulum is decreased after the reduction of membrane fluidity (*43*). The effect of phospholipid methylation on Ca^{2+} transport in human erythrocyte ghosts was also examined (*42*). Erythrocyte ghosts containing SAM increased the efflux of CA^{2+}, but the passive exchange of Ca^{2+} was not influenced by methylation.

Phospholipid Methylation and Arachidonic Acid Release

Histamine is released from basophils upon stimulation of the IgE receptor complex with an antigen (*44*). In studies of the role of phospholipid methylation in histamine release in RBL cells, close association between phospholipid methylation and arachidonic acid release from phospholipids was found (*45*). The RBL cells were first incubated with IgE and [^{3}H]methionine; then they were washed and stimulated with the IgE-specific antigen, ovalbumin. Within 1 minute after treatment of the sensitized cells with ovalbumin, a small increase in the incorporation of [^{3}H]methyl group into phospholipids was observed (Fig. 5). The increased methylation peaked in about 4 minutes, and after 5 minutes there was a decline in the [^{3}H]methyl group present

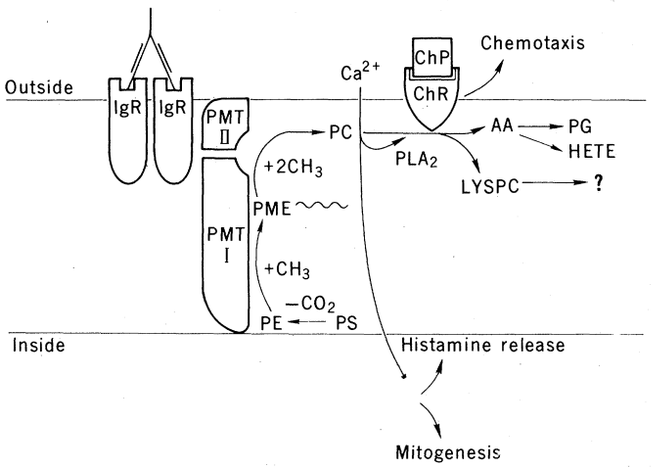

Fig. 4. Phospholipid methylation, Ca^{2+} influx, and phospholipase A_2 activation and transmission of biological signals through membranes. See text for explanations. IgE receptor (*IgR*); phospholipid methyltransferase I (*PMT I*); and phospholipid methyltransferase II (*PMT II*); phosphatidylserine (*PS*); phosphatidylethanolamine (*PE*); phosphatidyl-*N*-monomethylethanolamine (*PME*); phosphatidylcholine (*PC*); phospholipase A_2 (*PLA$_2$*); arachidonic acid (*AA*); prostaglandin (*PG*); lysophosphatidylcholine (*LYSPC*); 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (*HETE*); chemotactic peptide (*ChP*); chemotactic receptor (*ChR*).

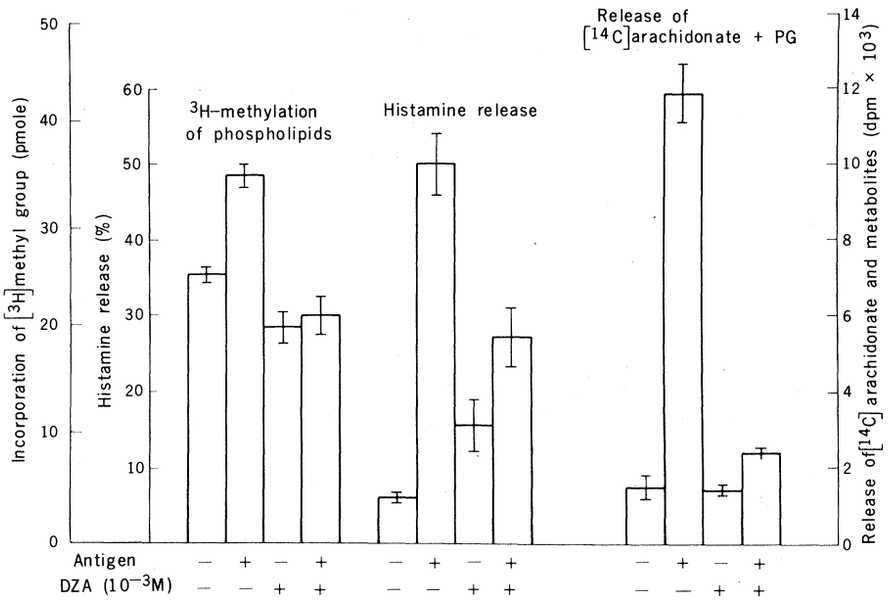

Fig. 5. Phospholipid methylation, arachidonic acid, and histamine release in basophilic leukemic cells (RBL). The cells were first incubated with [^{3}H]methionine or [^{14}C]arachidonic acid and ovalbumin-specific IgE. The cells were stimulated with ovalbumin and then the cells and supernatant fluid were assayed for [^{3}H]methyl groups incorporated into phospholipids, [^{14}C]arachidonic acid and prostaglandins (*PG*) generated from phosphatidylcholine and histamine; in paralleled experiments, cells were also treated with the methyltransferase inhibitor, 3-deazaadenosine (*DZA*). [From (*45*)]

Table 1. Phospholipid methylation, arachidonic acid release, and signal transduction in a variety of cell types.

Cell type	Stimulus	Phospholipid methylation	Arachidonic acid release	Biological effect
Rat reticulocyte	β-Adrenergic agonists	+	?	Cyclic AMP
C_6 glioma astrocytoma	β-Adrenergic agonists	+	+*	Cyclic AMP
	Benzodiazepine agonists	+	?	?
HeLa cells	β-Adrenergic agonists	+	+*	Cyclic AMP
Mast cells	Con A IgE receptor	+	+	Ca^{2+} influx, histamine release
Leukemic basophils	IgE specific antigens	+	+	Histamine release
Lymphocytes	Con A	+	+	Ca^{2+} influx, mitogenesis
Neutrophils	Chemotactic peptides	−	+	Chemotaxis
Fibroblasts	Bradykinin†	+	+	Cyclic AMP
Platelets	Thrombin, epinephrine‡	No effect	No effect	Aggregation

*After long-term stimulation. †F. Hirata and V. Manganiello (*56*). ‡A. Hotchkiss and N. R. Shulman (*57*).

in the phospholipids. Incorporation of [^{3}H]methyl group into lipids in unstimulated cells increased at a constant rate. Antigen-stimulated histamine release from RBL began to appear after 5 minutes and reached a maximum at 70 minutes. The release of histamine closely corresponded in time with the decline in incorporation of [^{3}H]methyl group into phospholipids. Thus, IgE-mediated histamine release appeared to be linked to the further metabolism of methylated phospholipids. After antigen stimulation, [^{3}H]methyl-labeled lysophosphatidylcholine was found indicating that phosphatidylcholine is cleaved by phospholipase A_2 to form lysophosphatidylcholine and a fatty acid. This possibility was examined by measuring the release of the fatty acid, arachidonate, by antigens after its incorporation into phospholipids. After RBL cells were incubated with [^{14}C]arachidonic acid, the extent of incorporation of the fatty acid into phospholipids was determined. Phosphatidylcholine contained most of the arachidonic acid (80 percent) incorporated into the phospholipids. Simulating RBL cells with ovalbumin resulted in the release of [^{14}C]arachidonic acid from phospholipids (Fig. 5) and a metabolite tentatively identified as prostaglandin D_2. The release of arachidonic acid closely paralleled the liberation of histamine from cells (*45*).

The relation between phospholipid methylation and the release of histamine and arachidonic acid from RBL cells was further established by using the methyltransferase inhibitor 3-DZA (*34*). After antigen stimulation the inhibition of phospholipid methylation and histamine release was almost identical at all concentrations of transferase inhibitors used (*45*); 3-DZA also blocked the release of [^{14}C]arachidonic acid from previously labeled phosphatidylcholine after stimulation with ovalbumin (Fig. 5). These findings indicate that phospholipid methylation and the release of arachidonic acid and histamine are closely coupled (Fig. 4). The activation of the IgE complex by antigen increases the methylation of phospholipids and may decrease membrane viscosity. When phospholipids are successively methylated, they are translocated in the membrane. This could bring the substrate phosphatidylcholine in juxtaposition to phospholipase A_2, an enzyme that catalyzes the hydrolysis of phosphatidylcholine to arachidonic acid and lysophosphatidylcholine (Fig. 4). The metabolism of the methylated phospholipid to arachidonic acid by phospholipase A_2 would then be facilitated. Arachidonic acid or its metabolites probably participates in the release of histamine since inhibition of phospholipase A_2 by mepacrine blocks the release of the biogenic amine (*45*). Phospholipid methylation also facilitates the entry of Ca^{2+}, a cation necessary for phospholipase A_2 activity (*37*), into mast cells (*40*). Arachidonic acid could arise from other phospholipids, such as phosphatidylinositol. The molecular mechanisms whereby arachidonic acid and its metabolites and lysophosphatidylcholine control the exocytotic release of histamine remain to be established.

Phospholipid Methylation, Arachidonic Acid, and Lymphocyte Mitogenesis

From the experiments described above it became apparent that phospholipid methylation affects several receptor-associated membrane events. This prompted a study of other cell systems involving signal transduction through membranes, such as lymphocyte mitogenesis. An important aspect of lymphocyte mitogenesis concerns the changes initiated in membrane binding of lectins and mobility of receptors on cell surfaces (*46*).

Mouse lymphocytes prepared from spleen were incubated with [^{3}H]methionine and then stimulated with Con A (*6*). The addition of the lectin caused about a doubling in phospholipid methylation. This increase in methylation peaked at 10 minutes and then returned to control levels in about 40 minutes (*6*). About 48 hours after the Con A treatment there was an increase in DNA synthesis as measured by [^{3}H]thymidine incorporation into DNA. By plotting dose response curves for Con A we found that low concentrations of the lectin stimulated and higher concentrations inhibited both phospholipid methylation and mitogenesis. The curves for phospholipid methylation and mitogenesis in lymphocytes were almost parallel, suggesting an association between the two events. Further evidence supporting this was the specificities of other lectins. The mitogenic lectins from *Wistaria floribunda* and *Pisum sativum* caused a transient increase and decrease in phospholipid methylation, whereas the nonmitogenic lectins from *Wistaria floribunda* agglutinin and *Bauhinea purpurea* had little effect (*6*).

Concanavalin A stimulated phospholipid methylation in T lymphocytes, but not in B cells (*6*). Lymphocytes obtained from spleens of athymic mice, deficient in T cells, did not respond to Con A by an increase in phospholipid methylation or thymidine incorporation. Destroying T cells in normal splenocytes with antibody to θ-receptor and complement abolished the stimulation of phospholipid methylation by Con A. When B cells in normal spleen cultures were selectively destroyed with antibody to immunoglobulin G and complement, phospholipid methylation was not affected.

The involvement of phospholipase A_2 in lymphocyte mitogenesis was examined by measuring the liberation of [^{14}C]arachidonic acid after its incorporation into phospholipids. Stimulation of lymphocytes with Con A resulted in a release of [^{14}C]arachidonic acid and small amounts of its metabolites, prostaglan-

dins E_2, F_2, and B_2 (*6*). Additional evidence that Con A stimulates phospholipase A_2 was the increased accumulation of [^{3}H]lysophosphatidylcholine. Indomethacin, an inhibitor of prostaglandin synthesis via the cycloxygenase pathway (*47*), did not affect lymphocyte mitogenesis, suggesting that other metabolites of arachidonic acid arising from the lipoxygenase pathway might be involved in mitogenesis.

Also useful for examining the relation between phospholipid methylation and the inhibition of mitogenesis in lymphocytes is the methyltransferase inhibitor 3-deaza-SIBA (*34*). A close correlation between inhibition of phospholipid methylation and thymidine incorporation in lymphocytes was shown at concentrations of the inhibitor that block lipid methylation, but not nucleotide methylation or the binding of ^{3}H-labeled Con A to lymphocytes (*6*). There was a considerable decrease in arachidonic acid release after inhibition of phospholipid methylation indicating that the fatty acid release is closely associated with the methylation of phospholipids. An influx of Ca^{2+} is a necessary component of the lymphocyte mitogenesis process (*48*), and the inhibition of phospholipid methylation appears to block lectin-stimulated influx of this cation. All of these findings indicate that phospholipid methylation, possibly by changing membrane fluidity, Ca^{2+} influx, and phospholipase A_2 activity, is an important biochemical event for the subsequent mitogenesis in lymphocytes (Fig. 4).

Arachidonic Acid Release, Phospholipid Methylation, and Chemotaxis

The interaction of membrane receptors with chemotactic peptides generates a directed movement of rabbit neutrophils (*49*). Chemotaxis of macrophages is blocked by methyltransferase inhibitors (*50*). These findings led to a study of the role of phospholipid methylation in chemotaxis of rabbit neutrophils (*8*). Neutrophils were incubated with L-[*methyl*-^{3}H]methionine and then stimulated with the potent chemoattractant peptide, fMet-Leu-Phe (fMet, formyl methionine; Leu, leucine; Ala, alanine). Within minutes there was an apparent decrease in incorporation of [^{3}H]methyl group into phospholipids (*8*). Similar results were obtained in guinea pig macrophages (*51*). This decreased methylation was observed with other chemoattractant peptides but not with antagonists (*8*). The decreased incorporation of [^{3}H]methyl group in lipids could be attributed to inhibition of methylation or to a greater rate of degradation of the methylated lipid. When the [^{3}H]methionine was removed with excessive amounts of unlabeled methionine, the methylated phospholipids disappeared more rapidly after stimulation of neutrophils with chemoattractants (*8*). This suggested that the peptides increase the degradation of the methylated phospholipid.

Phosphatidylcholine, the major methylated phospholipid, is synthesized either by the transmethylation (*3*) or the cytidine diphosphate (CDP)-choline pathways (*52*). To establish whether the phosphatidylcholine synthesized by the CDP-choline pathway is also affected by chemoattractants, we used [^{14}H]choline incorporated into the phospholipid of rabbit neutrophils. Despite the fact that much larger amounts of phosphatidylcholine were synthesized from [^{14}C]choline than [^{3}H]methionine, the phospholipids containing [^{14}C]choline did not respond to the chemoattractant peptide (*8*). This indicated that although the phospholipid formed by the transmethylation represents a small pool, it is much more metabolically active.

The peptide-stimulated degradation of methylated phospholipid could be a consequence of the activation of phospholipase A_2. [^{14}C]Arachidonic acid was therefore incorporated into phosphatidylcholine and rabbit neutrophils stimulated with fMet-Leu-Phe. There was a sharp release of [^{14}C]arachidonic acid and an accumulation of lysophosphatidylcholine (*8*). When a variety of chemoattractant peptides was tested, the order of their potency in stimulating the release of [^{14}C]arachidonic acid was paralleled by their capacity to promote chemotaxis. To further establish the relation between chemotaxis and the activation of phospholipase A_2 in neutrophils, we use inhibitors of the enzyme. The antimalarial drug mepacrine, a phospholipase A_2 inhibitor (*53*), blocked the release of arachidonic acid and chemotaxis in neutrophils in a dose-dependent manner (*8*). Another phospholipase A_2 inhibitor, 21-phosphohydrocortisone, also inhibited the release of the fatty acid and reduced chemotaxis (*8*). Inhibition of phospholipid methylation reduced the release of arachidonic acid and blocked chemotaxis. These findings indicate that phospholipid methylation and phospholipase A_2 activation are necessary components for neutrophil chemotaxis, as in the case of histamine release from mast cells (*7*) and basophils (*45*), and lymphocyte mitogenesis (*6*). Phospholipid methylation appears to be closely coupled to arachidonate release in neutrophil chemotaxis. Hardly any of the prostaglandin metabolites were generated after stimulation by a chemotactic peptide, which suggests that other metabolites of arachidonic acid arising from the lipoxygenase pathway may play a role in chemotaxis (Fig. 4).

In view of the importance of phospholipase A_2 in many membrane-associated events, we initiated a study of its regulation. An endogenous inhibitor of phospholipase A_2 in rabbit neutrophils was identified. This inhibitor is a protein of a molecular weight of about 40,000 and its synthesis is stimulated by glucocorticoids (*54*).

Conclusions

The asymmetric distribution of phospholipid methylating enzymes in membranes provides an important mechanism for the transmission of biochemical signals in cells. The topology of these enzymes makes possible the translocation of phospholipids from the cytoplasmic side to the outer surface of membranes by successive methylation. During the transit of phospholipids the viscosity of the membrane is reduced and this affects many membrane events. Catecholamine neurotransmitters, peptides, and immunoglobulins interacting with cell surface receptors initiate a cascade of biochemical and physical changes in local domains of the membrane. This leads to increased mobility of receptors, elevated phospholipid methylation, the generation of cyclic AMP, histamine release, mitogenesis, and chemotaxis. Not all receptor mediated events involve phospholipid methylation. Stimulation of platelets with thrombin, prostaglandins, or epinephrine had no effect on phospholipid methylation. Each type of receptor appears to have its own complement of phospholipid methylating enzymes to form a "signal cluster" (Fig. 4). Binding of a specific ligand with its receptor stimulates phospholipid methylation in a local area in the membrane changing the microenvironment in such a way as to facilitate the lateral mobility of the receptor complex (Fig. 2).

In several cell types examined, inhibition of phospholipid methylation blocks Ca^{2+} influx, the release of arachidonic acid, and the formation of lysophosphatidylcholine. Phospholipid methylation is closely coupled to phospholipase A_2, a Ca^{2+} requiring enzyme. This enzyme is a branch point for the formation of arachidonic acid and lysophosphatidylcholine (Fig. 4). Arachidonic acid serves as a

precursor for cycloxygenase and lipoxygenase enzymes (55). Cycloxygenase generates prostaglandins and lipoxygenase synthesizes hydroxy and hydroperoxy lipids. Inhibition of phospholipid methylation blocks the synthesis of these biologically important metabolites. Table 1 summarizes the activation of phospholipid methylation and arachidonic acid release in various cell types and the signals generated. These findings point to phospholipid methylation as an initial common pathway for the transduction of many receptor mediated biological signals through membranes.

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58. We thank F. T. Crews, K. Ishizaka, T. Ishizaka, E. Schiffmann, R. P. Siraganian, W. J. Strittmatter, S. Toyoshima, J. Tallman, and M. Waxdal for their collaboration in many of the experiments described.