Technical

Phospholipid Structure as a Modulator of Intracellular Turnover

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May I first say how pleased and honored I am to be the recipient of the Society's Award in Lipid Chemistry. My work has been in the field of phospholipid biochemistry and it is gratifying to follow a number of illustrious previous recipients who have done so much for this particular field. Eric Baer, the very first scientist to receive the award, and who, of course, worked in Toronto, laid the foundations for the chemical synthesis of phospholipids with defined stereochemical configurations. Then Herbert Carter in 1966, Gene Kennedy in 1970 and last year's awardee, Laurens van Deenen, have done monumental work in defining the chemical structure, metabolism and organizational role of these entities in biological membranes.

When I was invited to give this lecture, I was asked to talk on any subject of my choosing so I have decided to refer to past happenings only rather briefly and then only as a basis for discussing recent research and its implications for phospholipid turnover. When I began my scientific career soon after World War II, radioactive isotopes were just becoming available for research purposes. Inspired by reading Schroenheimer's classic work on the dynamic state of the body constitutents (1), I began to inject phospholipid precursors such as [^{32}P] phosphate into laboratory animals. It was still surprising to confirm the findings of pioneers like Artom, Chaikoff and Hevesey of a few years previously that when inorganic [^{32}P] phosphate was administered intraperitoneally, there was a fairly rapid uptake into the phospholipids extracted from brain. Thus, such phospholipids could no longer be considered as dull structural components of tissues with little metabolic activity but were, in fact, constantly being synthesized from the newly injected isotopic precursor. It also meant that in the adult, in a nongrowth situation, there must be a catabolism of equal magnitude. I remember making the calculation that, in mice, if one corrected for the impermeability of the blood-brain barrier, an amount of phosphorus equivalent to the total contained in the phospholipid of the brain would be exchanged in 70 hours (2). This seemed very surprising at the time and was doubted by many of my medical colleagues but, of course, as methods were developed for fractionating off the much more slowly turning over myelin component and for examining individual phospholipids, much faster times for the half-life of membrane phospholipids became apparent.

This continual turnover of the phospholipids in mammalian tissues fascinated me as a young biochemist and has done so ever since. It is still a highly puzzling phenomenon and none of the many explanations advocated for its necessity are convincing. The energy involved in the de novo formation of phospholipid molecules is considerable; we have recently calculated that 6-8% of the resting O_2 consumption of a rat is required to maintain phospholipid turnover.

Subcellular fractionation of tissues after isotopic labeling clearly showed that all the major membranes of the cell participate in the turnover. Yet in a normal cell, all of the isolated membranes apart from the endoplasmic reticulum both rough or smooth are incapable of synthesizing the bulk of the phospholipids which they contain. This dilemma was solved in experiments in which phospholipid-labeled microsomes were isolated from liver cells and incubated with unlabeled mitochondria. Upon reisolating the mitochondria after incubation in buffer, they gained no radioactivity unless one included in the system cytoplasmic protein (3,4). Similar conclusions were reached with labeled mitochondria and unlabeled microsomes. We now know from the brilliant and sustained studies of many groups, especially those of Drs. Wirtz and Zilversmit, that the cytoplasm contains a variety of exchange proteins which are believed to function in vivo by acting as carriers of newly synthesized phospholipids to any part of the cell where they are required to support dynamic turnover. To some extent, transfer of phospholipids can also be by lateral diffusion within a membrane bilayer system and work involving intraneural injection of [^{3}H] choline and electron microscopic autoradiography showed that this is the way that the myelin sheath of nerves is labeled from the Schwann cell plasma membrane (5).

All this implies that the synthetic capacity of the endoplasmic reticulum is, in reality, a back-up factory for supplying the phospholipid needs of all the membranes of the cell such as the nuclear, plasmalemma, lysosomal, Golgi, inner and outer mitochondrial membrane, as well as the requirements of the endoplasmic reticulum itself. It has long been suggested by reviewers that the phenomenon of turnover is necessary for the repair and maintenance of phospholipid molecules in membranes—now such a statement means that the molecules have to be damaged in some way or there would be no need for them to be replaced. In this respect, the phospholipases which are present in virtually all cell membranes would be candidates for bringing about this destruction; however, it seems most unlikely that the membranes would have a built-in capacity for destroying their own healthy phospholipid molecules unless this turnover was required for some other purpose. Nevertheless, because of their wide-spread distribution throughout the cell membrane system, one probably has to look on the phospholipases as the initiators of general phospholipid

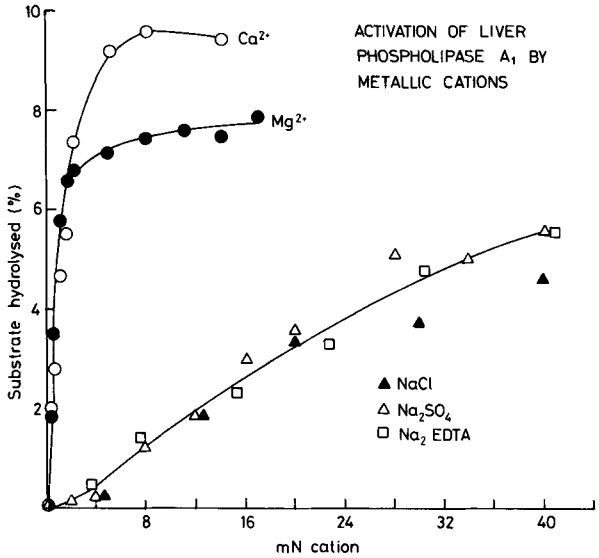

FIG. 1. The activation by various metallic cations of liver cytoplasmic phospholipase A_1 hydrolyzing a pure phosphatidylethanolamine substrate.

turnover. There are, of course, an increasing number of instances where phospholipases appear to be activated during defined physiological events in the cell. The dramatic response of the phosphoinositides to cell stimulation is an example and, here again, the initial event is probably a catabolic one, the localized loss of the inositol lipids causing a turn-on of the synthetic system, resulting in an enhanced labeling of certain membranes (6).

It is apparent, therefore, that the control of phospholipase action is likely to be of paramount importance, not only in regulating general turnover, but also in either turning on, or of equal importance—turning off phospholipid breakdown when, for example, a receptor is occupied and then vacated by an agonist. For the remainder of this talk, I want to discuss this control in terms of the theory (7), as yet unproven, that it is the state and environment of the lipid in the membrane that to some extent controls and regulates the enzymic activity of the phospholipases and, consequently, the synthesis and overall turnover.

To illustrate the two types of phospholipase action, I shall be referring to two cytoplasmic enzymes with which we have worked recently. The first is an active phospholipase A_1 which we have recently purified from liver cytoplasm but which is also present in brain, kidney and other tissues. It is likely that it may also be present in many membranes of the cell as well as the cytoplasm. At first sight, this phospholipase appears to be rather specific for phosphatidylethanolamine as a substrate, and to require, like many phospholipases, calcium for activity, but both conclusions are misleading. It was found that the enzymic activity was initiated not only by calcium but also by magnesium, strontium or barium. Surprisingly, even sodium EDTA could activate the reaction to a limited extent (Fig. 1). This stimulation could be attributed to the sodium added to neutralize the EDTA and the solution showed similar activation on a univalent cation basis to that observed if sodium chloride, sodium sulfate, or potassium chloride were added to the system.

It is fairly certain that these activations can be explained not in terms of a need for these cations as coenzymes, but as a requirement for the correct electrostatic condition at the interface. Our studies of some years ago showed that the Zeta potential at the plane of shear was often an active determinant of phospholipase activity (8). Now, at the pH used to measure enzymic activity, a phosphatidylethanolamine substrate particle would be expected to be negatively charged due to deprotonization of the amino group of the zwitterion. It seemed likely, therefore, that the metallic cations were acting as counterions, reducing the negative Zeta potential of the phospholipid-water interface and allowing the negatively charged enzyme which was operating above its isoelectric point (pI 7.2) to overcome the electrostatic energy barriers, preventing its active center combining with the substrate. Unfortunately, because of the small size of the substrate particles, it was difficult to confirm this supposition by measuring their mobility in an electric field and viewing under the optical microscope. It can be predicted that if the negative Zeta potential is lowered by adding a long-chain ionized cation to the substrate to reduce the surface potential, hydrolysis should be initiated in the absence of metallic cations. Indeed, stimulation of the reaction occurred upon introducing graded amounts of cetyl trimethylammonium bromide or cetyl pyridinium bromide into the substrate.

Now, if one considers the internal environment of the cell in which the enzyme operates, then the high intracellular concentrations of K^+ and appreciable Mg^{2+} present are fully sufficient to activate the enzyme in the test tube to its maximal activity. So, to prevent the cell membranes from undergoing spontaneous autolysis, some other control mechanism must be involved. A clue to a possible mechanism came when we admixed phosphatidylcholine with the substrate. This produced a dramatic inhibition of the hydrolysis such that, by the time 50% molar addition had been achieved 91 PC molecule/2 PE), inhibition was substantially complete.

It is known from recent studies using X-ray diffraction and ^{31}P nuclear magnetic resonance (NMR) that aqueous suspensions of phosphatidylethanolamine at temperatures above 10 C exist not as bilayer, but predominantly as the hexagonal II phase in which the polar head groups form inverted micelles and the hydrocarbon chains are in the continuous phase (Fig. 2) (9). Presumably the outer surface of such phospholipid structures must be covered with a layer of phosphatidylethanolamine molecules orientated with their hydrophilic phosphoethanolamine head groups adjacent to the aqueous environment. It makes good sense to believe that the enzyme can only attack the substrate when it is in this form and, probably, the 1 acyl ester bond is more exposed than in a tightly packed bilayer. With lecithin, because of the zwitterionic nature of the head group and its dimensions, the organized structure displayed in an aqueous environment is the liposome or continuous bilayer. Thus, if lecithin is added to phosphatidylethanolamine, the ^{31}P NMR shows that the phosphatidylethanolamine is forced into the bilayer configuration and inhibition of the enzyme might be expected to occur.

Using soybean phosphatidylethanolamine, Hui and his colleagues (10) have charted an approximate phase diagram for hydrated PC/PE mixtures using ^{31}P NMR measurements. This describes the transition from hexagonal to lamellar (bilayer) phase via a transitional state—the so-called LIPS particles—which are clearly shown by freeze-etching

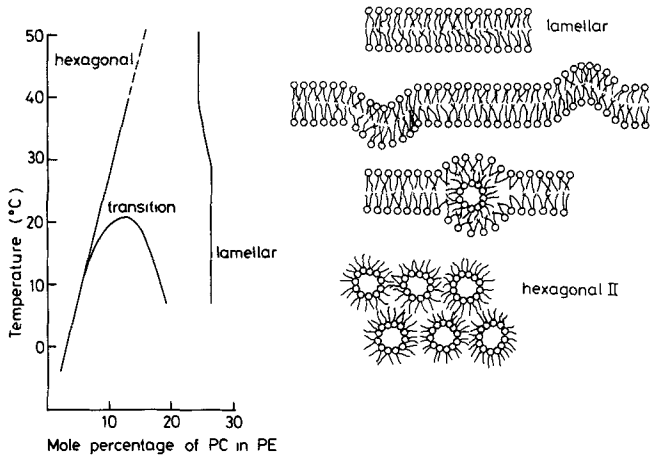

FIG. 2. Lamellar and hexagonal II forms of hydrated phospholipid particles in an incubation medium. The hydrophobic surface of the hexagonal phase would almost certainly be covered with an additional exterior layer of orientated molecules to make it more acceptable to the water phase. The phase changes produced upon adding PC to PE are illustrated on the left (10) and possible transitional forms (27) between lamellar and hexagonal II are on the right.

electron microscopy. These might consist of inverted micelles within the bilayer or, alternatively, the bulged or invaginated bilayer (Fig. 2). Clearly, in practice, the precise form of the phase diagram would depend on many things, e.g., the fatty acid composition of the phospholipids, the pH and the cation composition of the incubation medium.

In the phospholipase system, the prediction would be that at low PC/PE ratios, some of the PE would remain in the hexagonal phase and suffer enzyme attack, whereas at higher PC/PE ratios, inhibition would be complete. As a simple means of confirming these phase transitions of the substrate without the necessity of preparing large quantities of the phospholipid for ^{31}P NMR, we used electron microscopy with negative staining. Initially it was confirmed that the phospholipase would still operate in an incubation medium in which the system was activated by excess sodium ions and to which 0.5% phosphotungstate had been added as a negative stain. The pure phosphatidylethanolamine was clearly in the hexagonal phase with stacks of tubes, containing a lumen of phosphotungstate and showing end regions equivalent to a continuum of inverted micelles. Upon adding phosphatidylcholine sufficient to bring about almost total inhibition of the enzymic activity, electron microscopy indicated that all the phospholipid in the PE/PC mix was in the lamellar form.

Now let us consider the alternative situation in which phosphatidylcholine is the substrate. It might be expected that phosphatidylcholine would not be hydrolyzed by the enzyme, even if the active center could theoretically lock onto the susceptible ester bond of the substrate since it is almost totally in the bilayer configuation. However, if excess phosphatidylethanolamine is added to ^{32}P-labeled phosphatidylcholine, we find that labeled lysolecithin is produced at high PE/PC ratios. Presumably the excess PE is forcing some of the PC into the hexagonal phase. So, as has previously been pointed out for many other phospholipases, the apparent high degree of substrate specificity is not because of an inability to form an enzyme substrate complex but is a physicochemical phenomenon (11).

What is the message of all this for our understanding of the control of the phospholipase in relation to its attack on cell membranes? First, in a cell membrane, the predominant phospholipid is almost without exception phosphatidylcholine and one might expect, assuming random mixing, that the membrane lipids would be largely in the bilayer configuration. Thus, the phospholipids would be immune to attack. One can speculate, however, that under certain circumstances, domains would be produced in biological membranes which are richer in lipids not favoring the bilayer form and, indeed, recent ^{31}P NMR examinations have suggested this might be so. Such studies point to a small percentage of the phospholipids undergoing isotropic motion at 37 C (12,13). Although a number of explanations are possible, one of these would be that nonbilayer phases can exist in biological membranes. Such a phase change might be produced by a separation of anionic lipids possibly produced by local concentrations of agents such as calcium or by particular proteins. A further possibility is that the process of autoxidation of phospholipid molecules, which in all probability can occur in vivo, could, by introducing polar groupings into the hydrophobic regions, perturb the packing of the hydrocarbon chains, disturb the bilayer and produce conditions favoring hydrolysis (14). Recently, it has been shown that lipid peroxidation can promote phospholipid flip-flop by partially converting the bilayer structure into a nonbilayer configuration (15) and a number of studies have shown that peroxidized phospholipids are more susceptible to phospholipase attack (16). It seems possible, therefore, that the enzyme could preferentially attack those very areas where the phospholipids need repair and maintenance. Thus, the autoxidized phospholipids would be hydrolyzed and removed before their build-up could produce the well-described cytotoxic effects of autoxidation, e.g., the inactivation of membrane enzymes, damage to cytochromes and the disruption of membrane structure.

We turn now to possible control mechanisms for the physiologically responsive phospholipases. There is no doubt that dramatic and rapid reductions in the concentrations of certain cell phospholipids, especially the phosphoinositides, can occur in response to stimulation of the cell surface. I do not wish to enter into the controversy as to whether such changes are involved in Ca translocation or prostaglandin release. In the platelet, for example, we recently carried out a balance sheet of [^{3}H] arachidonic acid labeling patterns after treatment of the cells with thrombin to cause aggregation (17). By percentage, the most startling loss of activity was in the phosphatidylinositol with a third being lost in 40 seconds, although in magnitude, that from PC was about the same. There was a concomitantly small incorporation into phosphatidic acid and ethanolamine plasmalogens and, of course, a steady production of the eicosanoids including the prostaglandins. This phosphatidylinositol response occurs widely in a variety of experimental situations where surface receptors are stimulated and generally, it has been observed that most of the receptors mobilize Ca as a second messenger (6). In the platelet, we have also observed a substantial and very rapid breakdown of triphosphoinositide upon thombin treatment, equivalent to that seen in the hepatocyte stimulated with vasopressin (18) and within the fly salivary gland in which secretion has been activated with 5-hydroxy

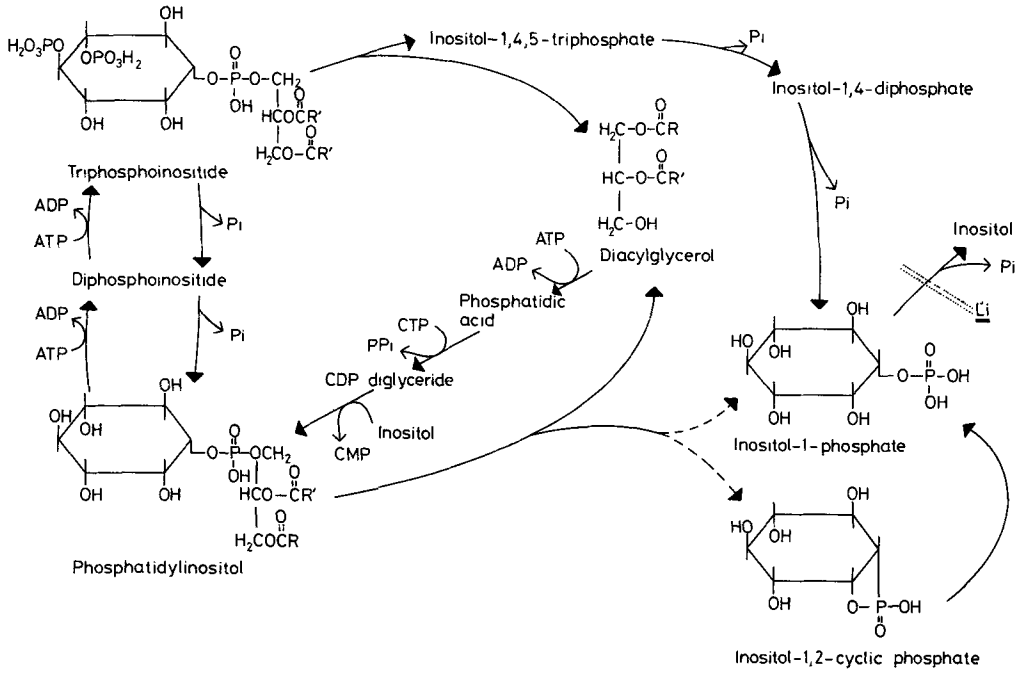

FIG. 3. The metabolism of phosphoinositides in tissues. The inositol-1-phosphatase is inhibited by lithium ions (28).

tryptamine (M. Berridge, unpublished data). At the moment, it is unclear whether this disappearance of polyphosphoinositides is acting as a back-up for phosphatidylinositol through the phosphomonoesterase system or whether there is an independent breakdown through phosphodiesterase action (Fig. 3). However, one result is abundantly clear—that in a cell in which the specific phosphatase hydrolyzing inositol-1-phosphate is blocked by lithium infusion, there is often an enormous increase in the concentration of inositol-1-phosphate and, to some extent, its cyclic derivative upon stimulation of the cell. This suggests that the phosphodiesterase hydrolyzing phosphatidylinositol into inositol phosphate and its cyclic derivative, as well as diglyceride, are stimulated (Fig. 3).

In virtually all tissues, we have shown that phosphatidylinositol phosphodiesterase exists as a powerful enzyme in the cytoplasm of cells, and whatever its participation in stimulus-response coupling, the problem remains why the membrane phosphatidylinositol is normally so stable to an enzyme with which it is in direct contact and which potentially could degrade it in seconds. Furthermore, if the enzyme is responsive to stimulation, what turns its activity on and off again in the stimulated-resting switch?

Recent work in our laboratory using the chromatofocusing technique already has established that the phosphodiesterase assayed at the generally reported pH optimum of 5.5 is very heterogeneous and a number of peaks of activity at different isoelectric points are apparent (19). However, if it is assayed at the more physiological pH of 7.0, only one sharp peak is visible with a pI of 4.6 and this is the activity we have investigated in the subsequent results described. Our observations on the enzyme have been carried out at a physiological pH and also in the presence of an 80-mM concentration of K^+ to mimic further the intracellular environment of the cell.

Preparations of the phosphodiesterase are almost totally inactive against all cell membrane preparations containing phosphatidylinositol prelabeled with [^{3}H] inositol unless the structure of the membrane is totally destroyed by deoxycholate addition or by sonication. Yet some of the phospholipid in the membrane would appear to be reasonably accessible at the membrane's surface since it can be degraded by other phospholipases, and part of it can readily exchange with phosphatidylinositol liposomes in the presence of a suitable exchange protein. It was surprising to find that, if the lipids were extracted from such cell membrane preparations, the labeled phosphatidylinositol in a dispersion of the lipids was again almost totally resistant to attack.

Investigations soon proved this was caused by the phosphatidylcholine present in such preparations. In fact, any phospholipids containing a phosphocholine hydrophilic head group, e.g., phosphatidylcholine, lysophosphatidylcholine and sphingomyelin, proved to be powerful inhibitors of phosphatidylinositol hydrolysis (20). In a liposome in which they were mixed in equimolecular proportions, inhibition was almost total. An organized water-lipid interface seemed to be essential for the inhibition because saturated lecithins with chain lengths down to C_8 were inhibitors of the reaction, whereas dihexanoyl lecithin was, in marked contrast, an excellent activator (21). The latter short-acyl chain lecithin caused a substantial break-up of the bilayer structure of the mixed liposomes as far as could be judged from electron microscopic and centrifugal studies.

We do not yet understand why a phosphocholine head group in juxtaposition to a phosphoinositol head group should prove to be such an anathema to the enzyme. However, the question of whether the phenomenon was truly occurring at a lipid-water interface was investigated using monolayers of [^{32}P] phosphatidylinositol, floating on a Langmuir-type trough. Some years ago, we had introduced the technique of following the phospholipase digestion of monolayers of such labeled phospholipids by measuring

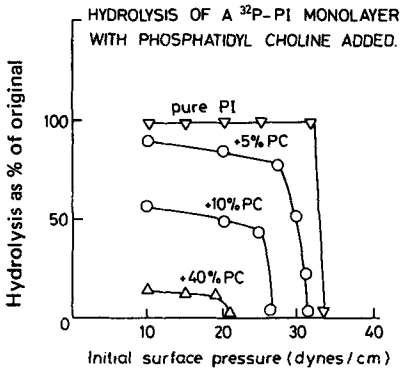

FIG. 4. Phosphatidylinositol phosphodiesterase inhibition by phosphatidylcholine. The activity is assayed by measuring the loss of surface radioactivity (22) when enzyme is injected under a monolayer of [^{32}P] phosphatidylinositol floating on buffer. The introduction of ovophosphatidylcholine brings about a progressive decrease of the maximal monolayer pressure, allowing hydrolysis and a reduction in the rate of hydrolysis.

the loss of surface radioactivity (22). When the phosphodiesterase was injected under a monolayer of [^{32}P] phosphatidylinositol, hydrolysis occurred until the film was compressed to 33 dynes/cm when an abrupt cut-off of activity occurred (Fig. 4). It was observed that a small percentage of phosphatidylcholine added to the film drastically reduced the threshold pressure, above which the enzyme would not operate (23). Thus, at 10% molar PC, this had decreased to 26 dynes/cm and at 40% molar, to 20 dynes/cm. Clearly the presence of the phosphatidylcholine made it more difficult for the enzyme to penetrate the surface and reach the susceptible ester bond of the phosphatidylinositol substrate. Even if the enzyme could penetrate at a lower pressure, there was always a substantial reduction in the rate of the hydrolysis compared to that occurring in the absence of phosphatidylcholine.

In contrast to the inhibitory action of choline-containing phospholipids, virtually all other membrane phospholipids were, to a greater or lesser degree, activators of the phosphodiesterase (20). Although in our test system, phosphatidylserine and phosphatidylethanolamine produced a 2.5-3.5-fold activation at 50% molar concentration, both phosphatidic acid and phosphatidylglycerol produced a similar stimulation in much lower proportions (10-15%). It was evident from monolayer experiments that phosphatidic acid was acting in a reverse way to phosphatidylcholine: graded additions were dramatically increasing the maximal film pressure of [^{32}P] phosphatidylinositol films which allowed penetration of the enzyme, so that by the time 15% molar phosphatidic acid had been added, hydrolysis occurred at the collapse pressure (23).

If one considers the membrane situation and, in particular, the plasmalemma in which the receptors are located, the phosphatidylinositol can be expected to be in a balanced sensitive state regarding its sensitivity to the phosphodiesterase activity. Although in the plasmalemma bilayer as a whole, there will undoubtedly be a preponderance of choline-containing lipids, it is likely that, in the inner cytoplasmic lipid leaflet, there will be a relatively higher concentration of excitatory lipids (24).

It is possible that our studies on the suppression and activation of phosphatidylinositol phosphodiesterase could provide a basis for understanding the sudden turn-on of phosphatidylinositol breakdown when a responsive cell is stimulated by an agonist. The receptor protein complexes presumably span the lipid bilayer of the plasmalemma and, when the agonist interacts with the exposed extracellular side of the protein, it is likely there would be a complimentary change of the intracellular side. This could be a conformational change, changing the hydrophobicity of the cytoplasmic end or the distribution and balance of its charged groups.

Such changes could, in turn, cause a phase separation in the inner leaflet of the bilayer lipids adjacent to the receptor complex, so that the acidic membrane phospholipids, including phosphatidylinositol molecules, would cluster around the receptor protein and become separated from the inhibitory choline phospholipids in the bulk-domain. In model systems, these clustering responses of acidic phospholipids are well known and can be induced by calcium or by hydrophobic or basic proteins (25,26). Once the cluster of acidic phospholipids became attacked by the cytoplasmic phosphodiesterase, diglyceride would be liberated. Since this diglyceride would often be rapidly rephosphorylated to phosphatidic acid by diglyceride kinase, the system is producing the very compound which would further catalyze the hydrolysis of phosphatidylinositol and the possibility exists of producing autocatalysis or self-amplification of the phosphatidylcholine hydrolysis.

To examine the quantities of phosphatidic acid required to produce such an effect, we prepared liposomes which mimicked the published analysis for the plasmalemma cytoplasmic leaflet of the bilayer (24). The introduction of 1 or 2 molecules of phosphatidic acid per 100 molecules of total phospholipid had a well defined stimulating effect on the breakdown of phosphatidylinositol in the liposome (21). In a cell membrane, once the self-amplification mechanism became operative, this could lead to a rapid breakdown of phosphatidylinositol. This would continue until either all available substrate was exhausted or until the agonist was removed and the transmembrane receptor protein reverted to its resting conformation and the inhibitory phospholipids came flooding back to damp down phosphatidylinositol hydrolysis (Fig. 5).

The process envisaged may seem speculative, but before much further progress can be made, we need to understand the molecular biology of how agonist-receptor interaction can often be followed within seconds by a turn-on of phosphatidylinositol hydrolysis and how and if this causes changes in the properties of the plasma membrane adjacent to the receptor. Already, phase changes in the lipids adjacent to receptors have been noted after stimulation by agonists, although at this stage it is not possible to deduce whether it is the cause or effect of the lipid hydrolysis which undoubtedly occurs.

To summarize, perhaps what I have been saying is the present indications are that at least some of the intracellular phospholipases are controlled by the choline-containing phospholipids in the membrane bilayer and that hydrolysis only occurs when the orderly, yet random, packing in a bilayer is perturbed by processes such as autoxidation or receptor function. This perturbation can either cause transient phase transitions or lateral phase separation in the bilayer. Once hydrolysis occurs, the system of phospholipid exchange within cells provides a ready means by which phospholipids synthesized in one

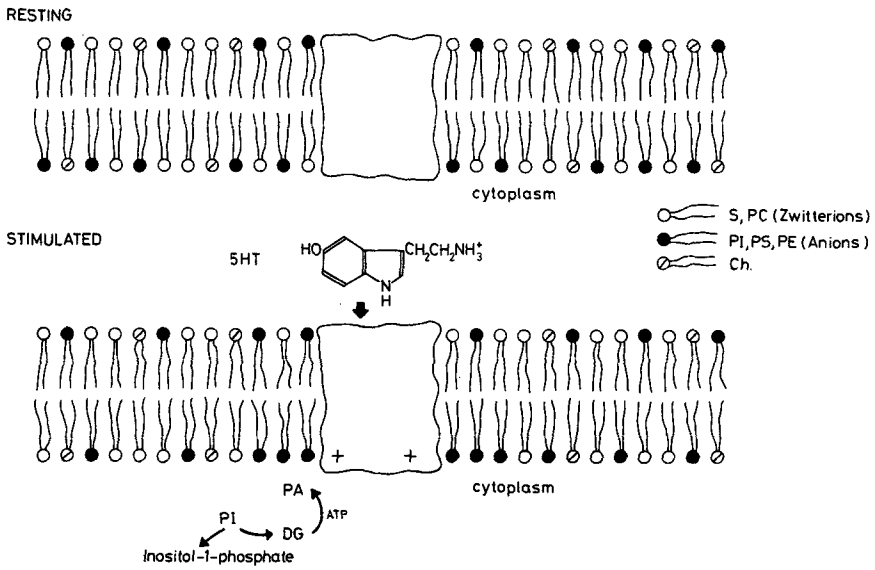

FIG. 5. Speculative diagram showing how receptor occupation by an agonist could cause the observed rapid hydrolysis of phosphatidylinositol. In the resting state, the phosphatidylinositol is protected from the action of the cytoplasmic phosphodiesterase by the zwitterionic phospholipids. Receptor stimulation by the agonist (in this example 5-hydroxytryptamine) causes a conformation change (charge or hydrophobic-hydrophilic balance) at the cytoplasmic end of the transbilayer receptor complex. This causes clustering of anionic phospholipids and allows phosphatidylinositol to be attacked. The diglyceride liberated can be phosphorylated to form phosphatidic acid which is a potent activator of the phosphodiesterase and could thus amplify the reaction.

membrane can pass to another membrane where depletion occurs. A fairly rapid turnover is perhaps essential to ensure that any local breakdown of the phospholipid bilayer due to metabolic or other deformation is quickly repaired in order to prevent such damage becoming lethal. The whole lipoprotein network of the cell can be considered an integrated complex which is in dynamic equilibrium, as far as phospholipids are concerned. Such a dynamic system would clearly be more capable of functional variation than one that is static.

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