The hydrolysis of phosphatidylinositol monolayers at an air/water interface by the calcium-ion-dependent phosphatidylinositol phosphodiesterase of pig brain

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1. The activity of Ca^{2+} -dependent phosphatidylinositol phosphodiesterase (EC 3.1.4.10) of pig brain against [^{32}P]phosphatidylinositol monolayers at an air/water interface has been measured. As the monolayer pressure was increased a sharp cut-off of enzymic hydrolysis occurred at $33 \times 10^{-3} \text{ N/m}$. 2. The addition of either phosphatidic acid, phosphatidylglycerol or oleyl alcohol increased the film pressure at which cut-off occurred, as well as increasing the rate of hydrolysis at lower pressures. 3. The rate of hydrolysis, but not the cut-off pressure, was markedly increased by oleic acid and slightly increased by phosphatidylethanolamine. 4. Phosphatidylcholine, palmitoylcholine and octadecylamine decreased the cut-off pressure, as well as the enzymic activity below this pressure. 5. Stearic acid and stearyl alcohol had no effect on either the cut-off pressure or the activity. 6. All activators decreased the length of the lag phase before enzyme activity began, and phosphatidylcholine increased it. 7. These results are compared with the stimulatory and inhibitory effects of various amphiphiles observed previously with phosphatidylinositol dispersions [Irvine, Hemington & Dawson (1979) *Eur. J. Biochem.* **99**, 525–530], and their possible relevance to the control of the phosphatidylinositol phosphodiesterase *in vivo* are discussed.

The hydrolysis of phospholipid monolayers at an air/water interface has been widely used as a model system to study the properties of phospholipases with respect to their regulation and mechanism of action (Bangham & Dawson, 1960; Quarles & Dawson, 1969; Quinn & Barenholz, 1975). Phospholipid monolayers are probably more similar to endogenous membrane substrates in their structure and dimensions than are lipid dispersions, and in addition are open to independent manipulation of the composition and packing density of their molecules. Also, sensitive monitoring of enzyme activity is possible by the disappearance of surface radioactivity (Bangham & Dawson, 1960; Gould & Dawson, 1972).

We have previously described a number of factors influencing the activity of the Ca^{2+} -dependent phosphatidylinositol-specific phosphodiesterase of rat brain assayed by using particles of the substrate (Irvine *et al.*, 1979; Irvine & Dawson, 1980a). In particular, we found phospholipids other than the substrate could markedly stimulate (for example phosphatidic acid, phosphatidylethanolamine) or inhibit (phosphatidylcholine, sphingomyelin) the activity. In addition, unsaturated amphiphiles such

as oleic acid and oleyl alcohol were stimulatory, and could be additive or even synergistic in their interaction with some of the phospholipids mentioned above (Irvine *et al.*, 1979).

These effects could be of great potential importance *in vivo*, as in addition to illustrating obvious regulatory mechanisms, they suggest a possible self-amplifying potential for phosphatidylinositol hydrolysis that may have physiological implications in the enhanced breakdown of phosphatidylinositol after agonist–receptor interaction (Irvine & Dawson, 1980a,b). It is essential therefore to clarify whether these effects of introducing other lipids into the phosphatidylinositol substrate are likely to occur in a membrane, or whether they are artefacts caused by dispersing or masking the substrate in water.

We have therefore here used a [^{32}P]phosphatidylinositol monolayer as a substrate for the phosphodiesterase, and have introduced the various activators and inhibitors mentioned above into the monolayer. By these means we have ascertained how they effect the monolayer properties, and in turn the activity of the phosphatidylinositol phosphodiesterase at various film pressures and packing densities of substrate molecules.

Materials and methods

Apparatus and monolayer techniques

The basic apparatus and techniques for the determination of the surface pressure and the surface radioactivity were essentially as described by Bangham & Dawson (1960).

The monolayer was formed on a circular poly-(tetrafluoroethylene) trough 6.8 cm in diameter and 1.1 cm deep kept at 35–37°C by a hotplate. The bulk aqueous phase (40 ml) in the trough was stirred continuously by a magnetic stirrer.

The surface radioactivity was measured with a mica end-window β -counter, which was mounted 5 mm above the monolayer film. The counter was connected to an integrating ratemeter (Panax) (Rikadenki Kogyo Co., Tokyo, Japan) and a continuous record of the surface count rate was obtained by using an integration time of 25 s. The surface tension was measured by a Wilhelmi plate (Zografi *et al.*, 1971); a grey-coated platinum plate (1 cm $\times$ 0.5 cm) dipping into the bulk phase was attached to the transducer of a microforce balance. A surface tension of $70\text{--}71 \times 10^{-3}$ N/m in the absence of phospholipid was taken as an indication that the platinum plate and liquid surface were clean.

The trough was filled with 0.02 M-maleate/KOH buffer, pH 7.0, containing 1 mM- CaCl_2 at 37°C. Phospholipids in light petroleum (b.p. 40–60°C) or chloroform solution were spread to form a monomolecular film and the pressure was allowed to stabilize (5–10 min). Where amphiphiles other than phosphatidylinositol were introduced into the monolayer, they were mixed with the substrate in the solvent solution before forming the layer. The hydrolysis was started by injecting pig brain extract containing 3 mg of protein (see below) under the monolayer.

Enzyme preparation

Fresh pig brain cerebral hemispheres (60–70 g) were finely chopped and homogenized in a Potter-Elvehjem homogenizer in 3 vol. of 25% (w/v) glycerol in water. The homogenate was centrifuged at 10000 *g* for 30 min, the supernatant filtered through glass wool and centrifuged again at 120000 *g* for 3 h at 0°C. The resulting supernatant was filtered through glass wool and then through approx. 10 g of dry silicic acid (Mallinckrodt) in a filter bed; it was finally centrifuged at 1000 *g* for 10 min. This final supernatant was colourless, free of phospholipids and showed a 5-fold enrichment on a protein basis over pig brain preparations made by the method of Irvine *et al.* (1979).

Presentation of data

The enzyme activity was defined as the proportional decrease of the initial surface radioactivity

3 min after the reaction had started. Results were standardized by the daily running of standard hydrolyses and are expressed as a percentage of these standard hydrolyses to allow for variation between different enzyme preparations. Each experiment was performed at least twice and the data are always representative.

Chemicals

[^{32}P]Phosphatidylinositol (10^5 d.p.m./ μg of phosphorus) was prepared from cultures of *Saccharomyces cerevisiae* grown in the presence of $^{32}\text{PO}_4^{3-}$ as previously described by Irvine *et al.* (1978). Phosphatidylcholine was purified from eggs as described by Dawson (1963). Phosphatidylethanolamine was purified from baker's-yeast phospholipids and phosphatidic acid was prepared from phosphatidylcholine using phospholipase D from celery by the method of Dawson & Hemington (1967). Stearic acid and octadecylamine were obtained from Sigma Chemical Co. (U.K.) and stearyl alcohol from Koch-Light (U.K.).

Palmitoylcholine was prepared by treating 1 g of choline chloride in 25 ml of dry pyridine with 3 ml of palmitoyl chloride under reflux for 3 h. The precipitate of palmitoylcholine that developed at 0°C was recrystallized from ethanol.

Fig. 1. Effect of the surface pressure of a [^{32}P]-phosphatidylinositol and phosphatidic acid mixed monolayer on phosphatidylinositol phosphodiesterase activity. Phosphatidylinositol monolayers contained various amounts of phosphatidic acid: ○, 0% (pure phosphatidylinositol); ●, 5%; △, 10%; ▲, 20%; □, 40% (as described in the Materials and methods section). Results were expressed as percentage of routine controls using pure phosphatidylinositol monolayers at 25×10^{-3} N/m and represent this activity plotted against the initial surface pressure of the film.

Results

General characteristics of the hydrolysis of phosphatidylinositol monolayers

Under the conditions specified, phosphatidylinositol monolayers had a collapse pressure of 48×10^{-3} N/m at 37°C . The Ca^{2+} -dependent phosphatidylinositol phosphodiesterase could not digest films above 33×10^{-3} N/m initial surface pressure (Fig. 1); at this point a sharp cut-off in enzymic activity occurred.

Throughout these experiments, a lag phase was observed between adding the enzyme and the commencement of hydrolysis; this was dependent on the initial surface pressure of the film (Fig. 2). The small peak seen in Fig. 2 for phosphatidylinositol monolayers at about 20×10^{-3} N/m was highly reproducible. At 32×10^{-3} N/m (maximum pressure at which hydrolysis occurred) the lag was 6.5 min, and at this pressure, each phosphatidylinositol molecule occupied 0.59 nm^2 (Fig. 3).

Fig. 2. The relation between lag phase of enzymic hydrolysis and initial surface pressure of monolayer Phosphatidylinositol monolayers contained various amounts of phosphatidic acid [○, 0% (pure phosphatidylinositol); ●, 5%; △, 10%] or of phosphatidylcholine [□, 5%; ■, 10%; ○, 20%].

Effect of phosphatidic acid and phosphatidylglycerol

The collapse pressure of a pure phosphatidic acid film was 34×10^{-3} N/m and sequential addition of this phospholipid to phosphatidylinositol gradually lowered the collapse pressure towards this value (see Fig. 4). Phosphatidic acid slightly stimulated the rate of hydrolysis at all surface pressures, but its most pronounced effect was to increase the pressure at which the cut-off activity occurred; 20% allowed phosphatidylinositol hydrolysis at the collapse pressure (Fig. 1). Some shortening of the lag phase was also observed (Fig. 2).

Fig. 3 shows the effect of phosphatidic acid on the pressure/area-per-molecule curve of phosphatidylinositol. As the phosphatidic acid is increased, the number of phosphatidylinositol molecules per unit area at a given pressure was changed very little; this may be a consequence of the small head group of phosphatidic acid, which contributes comparatively only a small area, provided it is diluted by the phosphatidylinositol molecules, thus removing the

Fig. 3. Surface-pressure-area-per-phospholipid-molecule curves of phosphatidylinositol and phosphatidic acid mixed monolayers

Phosphatidylinositol monolayers contained various amounts of phosphatidic acid: ○, 0% (pure phosphatidylinositol); ●, 20%; △, 50%; ▲, 100% (pure phosphatidic acid).

Fig. 4. Effect of phosphatidylcholine and phosphatidic acid in phosphatidylinositol monolayers on the collapse pressure of the monolayer and the maximum initial surface pressure allowing enzyme activity

Phosphatidylinositol monolayers contained various amounts of phosphatidic acid or phosphatidylcholine. The results for maximum initial pressure allowing enzyme activity are derived from Figs. 1 and 6. $\circ$, Phosphatidic acid; Δ , phosphatidylcholine. Also plotted are the collapse pressures for phosphatidylinositol plus phosphatidic acid ($\bullet$) or phosphatidylcholine ($\blacktriangle$) monolayers.

greater charge repulsion between adjacent phosphatidic acid molecules.

Phosphatidylglycerol was very similar in its effect to phosphatidic acid, as it increased both the rate of hydrolysis and the cut-off point of enzyme activity (Fig. 5).

Effect of phosphatidylcholine and palmitoylcholine

Phosphatidylcholine monolayers had a collapse pressure of 49×10^{-3} N/m. The addition of this phospholipid to a phosphatidylinositol monolayer had a marked effect on the phosphodiesterase activity, as might be expected from previous experiments (Irvine *et al.*, 1979). The rate of hydrolysis at all pressures, and the pressures at which the cut-off in activity occurred, were decreased by addition of

Fig. 5. Effect of initial surface pressure of [32 P]phosphatidylinositol and phosphatidylglycerol mixed monolayers on phosphatidylinositol phosphodiesterase activity. Phosphatidylinositol monolayers contained various amounts of phosphatidylglycerol: ∇ , 0% (pure phosphatidylinositol); $\circ$, 5%; $\bullet$, 10%; $\blacktriangle$, 20%; Δ , 40%; $\blacksquare$, 50%. The data are expressed as percentages of routine controls using pure phosphatidylinositol as a substrate at 25×10^{-3} N/m (as in Fig. 1).

Fig. 6. Effect of initial surface pressure of [32 P]phosphatidylinositol and phosphatidylcholine or palmitoylcholine mixed monolayers on phosphatidylinositol phosphodiesterase activity

Phosphatidylinositol monolayers contained various amounts of phosphatidylcholine [∇ , 0% (pure phosphatidylinositol); $\circ$, 5%; $\bullet$, 20%; Δ , 40%] or palmitoylcholine ($\blacksquare$, 10%; $\square$, 25%). Data are expressed as percentages of controls using pure phosphatidylinositol at 25×10^{-3} N/m (as in Fig. 1).

phosphatidylcholine (Fig. 6). The lag phase was also increased (Fig. 2).

Palmitoylcholine showed similar effects to phos-

phatidylcholine, in decreasing both the cut-off threshold pressure and the rate of hydrolysis (Fig. 6).

Effects of phosphatidylethanolamine and octadecylamine

The effects of adding phosphatidylethanolamine and octadecylamine to a phosphatidylinositol monolayer were investigated, because they both have a positively charged amine residue in the hydrophilic head group. Octadecylamine has been reported to increase the threshold pressure at which phosphatidylinositol phosphodiesterase can hydrolyse phosphatidylinositol monolayers (Quinn & Barenholz, 1975) and phosphatidylethanolamine has a stimulatory effect similar to phosphatidic acid when the enzyme is attacking a dispersion of the substrate (Irvine *et al.*, 1979). However, the effect of phosphatidylethanolamine was not the same as phosphatidic acid in the monolayer, as although the enzyme activity was increased (Fig. 7), the pressure at which a cut-off in activity occurred was not altered. Monolayers of phosphatidylethanolamine alone had a collapse pressure of 35×10^{-3} N/m.

In contrast with phosphatidylethanolamine, octadecylamine inhibited both the enzyme activity and the maximum pressure of activity in a similar

manner to choline-containing lipids (Fig. 7). These results are in contrast with those of Quinn & Barenholz (1975), although these latter workers used a different preparation of the phosphodiesterase in their experiments. Also, at pressures above 40×10^{-3} N/m, we observed a non-enzymic hydrolysis of phosphatidylinositol in the presence of more than 50% octadecylamine. This is presumably caused by the close apposition of the positively charged amine head group to the diester linkage of phosphatidylinositol, which enables nucleophilic attack to occur (Davies & Rideal, 1961).

Effects of fatty acids and alcohols

By using a lipid dispersion as a substrate for the phosphatidylinositol phosphodiesterase, oleic acid activated the enzyme in an apparently different way from phosphatidic acid, as when mixed with this phospholipid the independent stimulations by each

Fig. 7. Effect of initial surface pressure of [32 P]phosphatidylinositol plus phosphatidylethanolamine or octadecylamine mixed monolayers on phosphatidylinositol phosphodiesterase activity

Phosphatidylinositol monolayers contained various amounts of phosphatidylethanolamine [∇ , 0% (pure phosphatidylinositol); $\circ$, 5%; $\bullet$, 20%] or of octadecylamine ($\triangle$, 10%; $\blacktriangle$, 40%). The data are expressed as percentages of routine controls using pure phosphatidylinositol monolayers at 25×10^{-3} N/m (as in Fig. 1).

Fig. 8. Effect of initial surface pressure of [32 P]phosphatidylinositol plus fatty acid or alcohol mixed monolayers on phosphatidylinositol phosphodiesterase activity

Phosphatidylinositol monolayers contained various amounts of stearic acid [∇ , 0% (pure phosphatidylinositol); $\circ$, 20%; $\bullet$, 40%], oleic acid ($\square$, 30%; $\blacksquare$, 50%), stearyl alcohol ($\triangle$, 20%; $\blacktriangle$, 40%), or oleyl alcohol ($\square$, 40%; $\blacksquare$, 50%). The results are expressed as percentages of routine controls using pure phosphatidylinositol as substrate at 25×10^{-3} N/m (as in Fig. 1).

Fig. 9. The relation between lag phase before enzymic hydrolysis and initial surface pressure of [^{32}P]phosphatidylinositol and oleic acid mixed monolayers

Phosphatidylinositol monolayers contained various amounts of oleic acid: ∇ , 0% (pure phosphatidylinositol); $\circ$, 30%; $\bullet$, 40%; $\triangle$, 50%; $\blacktriangle$, 60%. The length of the lag phase (see the text) of enzymic hydrolysis against initial flow pressure is plotted.

were additive and under some conditions synergistic (Irvine *et al.*, 1979). A possible explanation for this difference is apparent from the way oleic acid stimulates the hydrolysis of the phosphatidylinositol monolayer (Fig. 8). The rate of hydrolysis is greatly increased, yet there is no alteration in the pressure threshold allowing hydrolysis. The lag phase before hydrolysis commenced was decreased (Fig. 9).

Oleyl alcohol, which also stimulates the phosphodiesterase and which we had previously assumed to act in a manner similar to oleic acid (Irvine *et al.*, 1979), was in these experiments clearly different, as in addition to stimulating the activity it also increased the pressure at which the cut-off in activity occurred (Fig. 8); indeed, eventually, as with phosphatidic acid (Fig. 1), hydrolysis still took place at the collapse pressure when sufficient activator was added. Our previous observation that an unsaturated bond is necessary in long-chain amphiphiles to activate is confirmed here by the lack of effect of either stearic acid or stearyl alcohol in the rate of hydrolysis of the phosphatidylinositol monolayer (Fig. 8).

Discussion

This investigation clearly establishes that previous results, showing marked stimulations and inhibitions of soluble phosphatidylinositol phosphodiesterase by adding certain amphipathic substances to the substrate in aqueous dispersion (Irvine *et al.*, 1979) are truly surface phenomena and not artefacts produced by an alteration in the gross physical nature of the lipid dispersions. All the activators and inhibitors have effects on the hydrolysis of phosphatidylinositol molecules precisely orientated at an air/water interface, which can be extrapolated to confirm and extend the results obtained by the use of dispersions. Such results are likely therefore to be pertinent to the situation *in vivo* where the cytoplasmic phosphodiesterase of cells would be interacting with the substrate existing presumably as a bilayer, with its hydrophilic head group orientated to the enzyme in the same manner as with the monolayer. It should be noted, though, that the high calcium concentrations used here (compared with those pertaining *in vivo*), and the lack of membrane proteins in the pure lipid monolayer, mean that this system is still somewhat artificial.

The results obtained do, however, allow some limited insight into the mechanism of the activations and inhibitions observed. When a protein in general interacts with a phospholipid monolayer at an air/water interface, three phases can be recognized depending on the pressure applied to the film and consequently the packing density of its molecules (Quinn & Dawson, 1970). At very low packing densities whole protein molecules can penetrate and partially or wholly unfold at the air/water interface; at intermediate pressures certain groups of the protein can penetrate into the film with the bulk remaining in the aqueous phase; and at high film pressures the protein can interact with the phospholipid without penetration. Generally phospholipases cannot hydrolyse their substrates in a monolayer unless at least partial penetration occurs and this certainly seems to be true for the present phosphodiesterase, since for a pure phosphatidylinositol film there is a sharp cut-off point as the film pressure is increased to $33 \times 10^{-3} \text{ N/m}$. This is in the same region as the cut-off pressure observed for other phospholipases acting against different phospholipid substrates existing as monolayers (Bangham & Dawson, 1960, 1962; Dawson, 1966; Quarles & Dawson, 1969; Demel *et al.*, 1975). Such a pressure threshold will depend not only on the structure of the enzyme and the nature of its active centre but also on the fatty-acid composition of the phospholipid and the electrostatic condition appertaining at the lipid/water interface. The latter in turn will depend on the nature of the hydrophilic head group of the phospholipid and the ionic composition of the bulk phase. At pressures above the cut-off

point, it has often been observed that changing the electrostatic conditions (by introducing, for example, charged phospholipids into the film) could assist penetration of the active centre and induce enzymic activity.

However, this cannot be the entire explanation of the present observations. The phosphoinositol head groups of the substrate would themselves possess a negative charge. Although phosphatidic acid addition might be expected to increase the charge density somewhat [because of its two fully ionized negative sites per molecule (Hauser & Dawson, 1967)], phosphatidylglycerol would not effect the density, and oleyl alcohol would lower it. Yet all these substances when mixed with phosphatidylinositol are capable of inducing hydrolysis of the film even at the collapse pressure, although they are not as potent as phosphatidic acid. Clearly they are introducing discontinuities into the film packing that allow penetration of the active centre, even at high film pressures. Possibly at such pressures the closely packed inositol rings physically prevent penetration, whereas the smaller head groups of phosphatidylglycerol, phosphatidic acid and oleyl alcohol provide a route that allows the active centre of the enzyme to penetrate to the susceptible phosphodiester bond of the substrate. The steric factors involved must be complex and precise since, unlike oleyl alcohol, stearyl alcohol is not effective in this function. However, with the latter substance the mean area occupied by the orientated hydroxy group on the surface will be less than with the oleyl alcohol because of perturbation produced by the unsaturated link in the hydrocarbon chain. Presumably this factor is sufficient to determine whether adequate penetration of the active centre does or does not occur. The activators as a whole seem to represent wedge-shaped molecules with a fairly small head group compared with the effective cross section area of the hydrocarbon chains. In a lipid dispersion such molecules would have a tendency to induce the bilayer form of phospholipid packing to transform to a hexagonal phase (Cullis & DeKruijff, 1979).

Conversely, addition of phosphatidylcholine causes a marked fall in the pressure threshold for film hydrolysis. Although the zwitterionic phosphocholine head group will dilute out the surface charge density of the interface presented to the enzyme, this again cannot be the whole explanation since oleyl alcohol would do the same. Moreover, palmitoylcholine and octadecylamine, which would markedly decrease the density by surface-charge neutralization, are certainly no more effective at decreasing the pressure threshold than phosphatidylcholine. Presumably the phosphocholine head group, possibly lying planar to the surface (Phillips *et al.*, 1972), increases the steric factors preventing successful penetration.

The process of penetration of the active centre is a statistical phenomenon, which will depend on the collision velocity and orientation of the protein molecules and the energy barriers preventing penetration. It will thus depend initially on protein concentration, although once penetration has occurred it will not be reversed by decreasing the concentration (Quinn & Dawson, 1970). For some reason a critical penetration of protein is required before activity is observed, as all the enzymic hydrolyses showed a lag phase. All of the activators whether or not they assist penetration at films above 33×10^{-3} N/m decrease the lag phase before hydrolysis commences. What this critical build up is likely to be is not immediately clear; it could be that at a given point the release of diacylglycerol groups into the film acts as an autocatalytic centre for further hydrolysis.

The three agents described that allow penetration of the active centre of the enzyme into high-pressure films of phosphatidylinositol also decrease the lag phase, and increase (by 20–50%) the rate of hydrolysis of substrate films at pressures below 33×10^{-3} N/m. Either these activators increase the rate and extent of penetration even at low pressures or they have effects on the enzymic interaction itself. That the latter is possible is seen by the behaviour of oleic acid added to the substrate. This fatty acid does not in any way assist penetration into high-pressure films, yet it has a marked effect in increasing the rate of substrate hydrolysis below 33×10^{-3} N/m by 100–150%. The reason for this is not known, although the carboxylic acid group would have great affinity for the Ca^{2+} required by the enzyme and might put such ions into a favourable position for successful hydrolysis to occur. Again the interfacial steric requirements must be very precise because the absence of a double bond in the fatty acid (stearic acid) entirely eliminates its activating effect. Such independent effects of substances on the penetration and rate of hydrolysis could explain why in substrate dispersions the effect of activators can be additive and even synergistic (Irvine *et al.*, 1979). The activation produced by phosphatidylethanolamine seems to be a similar type to that produced by oleic acid in that it does not assist penetration at film pressures over 33×10^{-3} N/m. It increases the rate of hydrolysis of low-pressure films to a similar extent as the other activating phospholipids. Unlike phosphatidylcholine, its zwitterionic head groups would perhaps extend tangentially to the plane of the surface (Phillips *et al.*, 1972).

Although some of the substances studied do not occur in cell membranes the behaviour of those that do (the glycerophospholipids and fatty acids) has obviously physiological implications. It is generally agreed that in a cellular membrane the surface pressure of the bulk lipid phase is of the order of

$33 \times 10^{-3} \text{ N/m}$ (Demel *et al.*, 1975). It is obvious, then, that the marked effects of phosphatidylcholine and phosphatidic acid (a secondary product of phosphatidylinositol catabolism; Hokin, 1968) on the cut-off point of the enzyme's activity means these phospholipids could exert an extraordinarily tight control over the activity of the enzyme against a cellular membrane. Furthermore, once the phosphodiesterase is able to begin to attack its substrate because of a correct acidic-phospholipid/choline-phospholipid balance, then such a stimulation could be greatly amplified by the presence of an unsaturated fatty acid, another secondary product of catabolism (Irvine & Dawson, 1980b). In summary, therefore, the present results confirm and extend our suggestion that the composition and physico-chemical state of a membrane around a phosphatidylinositol molecule can exert a profound effect on its susceptibility to hydrolysis.

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