# Phosphatidylinositol-4,5-bisphosphate phosphodiesterase and phosphomonoesterase activities of rat brain

## Some properties and possible control mechanisms

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1. The phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)$P_2$] [and to a lesser extent, the phosphatidylinositol-4-phosphate (PtdIns4$P$)] phosphodiesterase and monoesterase activities of a rat brain supernatant have been studied by using $^{32}$P-labelled substrates prepared from human red blood cells. 2. PtdIns(4,5)$P_2$ monoesterase is maximally stimulated by $Mg^{2+}$, though some activity is detectable in $Ca^{2+}$/EDTA ($Mg^{2+}$-free) buffers. The phosphodiesterase, however, is $Ca^{2+}$-dependent, and in $Ca^{2+}$/EDTA buffers with the pure lipid as substrate, shows maximal activity at 100 nM-$Ca^{2+}$. 3. If PtdIns(4,5)$P_2$ is presented as a component of a lipid mixture of similar composition to that of the inner half of the lipid bilayer of a rat liver plasma membrane, the phosphodiesterase shows considerable activity at 1 μM-$Ca^{2+}$, and is maximal at 100 μM-$Ca^{2+}$. However, if it is assayed against the same substrate in $Ca^{2+}$/ EGTA buffers with 3 mM-$Mg^{2+}$ and 80 mM-KCl present (as an approximate parallel with the ionic environment *in vivo*), it shows no detectable activity below 100 μM-$Ca^{2+}$, and is maximal at 1 mM-$Ca^{2+}$. The monoesterase can hydrolyse PtdIns(4,5)$P_2$ in such a lipid mixture at all $Ca^{2+}$ concentrations with 1 or 3 mM-$Mg^{2+}$ present. 4. PtdIns(4,5)$P_2$ phosphodiesterase can be induced to attack its substrate under ionic conditions similar to those *in vivo* (0.1–1 μM-$Ca^{2+}$; 1 mM-$Mg^{2+}$; 80 mM-KCl) by the conversion of its substrate into a non-bilayer configuration. If given such a substrate [by mixing PtdIns(4,5)$P_2$ with an excess of phosphatidylethanolamine (PtdEtn)] it shows a shallow $Ca^{2+}$-dependency curve from 0.1 to 100 μM and then a steep rise to 1 mM-$Ca^{2+}$. Together these observations lead us to the suggestion that a perturbation in a membrane *in vivo* equivalent to a non-bilayer configuration would be sufficient to induce phosphodiesterase-catalysed PtdIns(4,5)$P_2$ breakdown. 5. When given substrates mixed with excess PtdEtn at pH 7.25 (or 5.5), 1 μM-$Ca^{2+}$, 1 mM-$Mg^{2+}$ and 80 mM-KCl, the rat brain supernatant phosphodiesterase activity hydrolysed PtdIns(4,5)$P$ 50–100-fold faster than it hydrolysed phosphatidylinositol (PtdIns). If the supernatant was presented with such a non-bilayer mixture containing a ten-fold excess of PtdIns over PtdIns(4,5)$P_2$, the latter phospholipid was still hydrolysed by phosphodiesterasic cleavage at nearly ten times the rate of the former. 6. Receptor-stimulated phosphodiesterase cleavage of polyphosphoinositides is an early event in cell activation by many agonists. The properties of PtdIns(4,5)$P_2$ phosphodiesterase *in vitro* suggest that a change in the presentation of its substrate would be a sensitive and sufficient control on the enzyme's activity *in vivo*.

Abbreviations used: PtdIns4$P$, phosphatidylinositol 4-phosphate; PtdIns(4,5)$P_2$, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)$P_3$, inositol 1,4,5-trisphosphate; Ins(1,4)$P_2$, inositol 1,4-bisphosphate; PtdIns, phosphatidylinositol; CDTA, *trans*-1,2-diaminocyclohexane-*NNNN*-tetra-acetic acid; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine.

The catabolism of the polyphosphoinositides PtdIns(4,5)$P_2$ and PtdIns4$P$ is catalysed by phosphodiesterase(s) and phosphomonoesterases found in probably all animal tissues (Dawson & Thompson, 1964; Hawthorne & White, 1975; Irvine, 1982). A stimulated catabolism of inositol lipids occurs in many tissues responding to a wide range

of agonists (Michell, 1975; Downes & Michell, 1982a; Abdel-Latif, 1983), and recent evidence suggests that the primary stimulated pathway is by phosphodiesterase action on $PtdIns(4,5)P_2$ and PtdIns4P, resulting in the formation of $Ins(1,4,5)P_3$, $Ins(1,4)P_2$ and diacylglycerol (Agranoff et al., 1983; Berridge, 1983; Berridge et al., 1983). Furthermore, it has been suggested that some (or all) of the disappearance of PtdIns that also occurs in these tissues is the result of phosphorylation of PtdIns to replace the polyphosphoinositides (Michell et al., 1981). The $Ins(1,4,5)P_3$ formed by phosphodiesterasic cleavage of $PtdIns(4,5)P_2$ may act as an intracellular messenger to release $Ca^{2+}$ (Streb et al., 1983) and the diacylglycerol so formed also has possible messenger functions (Kaibuchi et al., 1982). The phosphodiesterase(s) hydrolysing polyphosphoinositides have therefore assumed a central place in current concepts of the regulation of cell activation by many agonists, yet at present very little is known about how their activity may be controlled (Irvine, 1982).

Our recent studies on the phosphodiesterase which hydrolyses PtdIns (Irvine et al., 1979; Dawson et al., 1980; Hirasawa et al., 1981; Irvine et al., 1984) have demonstrated how possible regulatory mechanisms of phospholipases may be explored if the state of presentation of the substrate, or the ionic environment of the enzyme, are manipulated. PtdIns4P and $PtdIns(4,5)P_2$ phosphodiesterases and monoesterases have generally been studied with the addition of detergents (to stimulate activity) or with non-physiological ion concentrations (for example millimolar calcium-ion concentrations). Here we have attempted to mimic intracellular conditions more closely, as did Downes & Michell (1982b) for the membrane-bound $PtdIns(4,5)P_2$ phosphodiesterase of red blood cells, in order to study possible control mechanisms of these enzymes. A preliminary account of some of these data has appeared elsewhere (Irvine et al., 1984).

## Materials and methods

### Enzyme source

This was a rat brain supernatant prepared exactly as in Irvine et al. (1979). Several supernatants were used over the course of this work, and all were adjusted to the same protein concentration to give uniformity of results, though there were minor quantitative differences in absolute activities between preparations.

### [$^{32}P$]PtdIns

This was prepared as described by Irvine et al. (1978).

### Phospholipids

PtdSer and sphingomyelin were obtained from Sigma. PtdCho, PtdEtn and PtdIns were generally prepared from yeast (Irvine et al., 1978), though sometimes these lipids prepared from pig liver were employed; we found no difference between the same phospholipid from different sources in these experiments.

### [$^{32}P$]PtdIns(4,5)$P_2$ and [$^{32}P$]PtdIns4P

These were purified from $^{32}P$-labelled erythrocyte ghosts prepared as described by Downes et al. (1982). We modified their procedure only in substituting citrate for $Cl^-$ in the rejuvenating medium (and in the buffer used for the last red-blood-cell centrifugation), since this increases the $^{32}P$ incorporation (P. T. Hawkins, personal communication). Blood was obtained from the Cambridge Blood Transfusion Centre and was used at less than 2 weeks old. Labelling was for 2h before lysis of the cells, and the red-cell ghosts were usually stored overnight at 4°C between the third and fourth washes.

When the red-cell ghosts had lost all their colour by repeated washing, the packed membranes (approx. 100ml) were mixed with 375ml of chloroform/methanol (1:2, v/v) + 1ml of concentrated HCl, and two phases formed by the addition of 120ml of chloroform and 120ml of water. After centrifugation (500g), the lower phase was washed twice with Folch theoretical upper phase containing 1M-HCl. The lower phase was then washed three times with theoretical upper phase containing 0.04% $CaCl_2$ in the water to convert inosites into their calcium salts. The lower phase was dried down, resuspended in chloroform and loaded on to a column (30g) of silicic acid. $PtdIns(4,5)P_2$ and PtdIns4P could be quantitatively eluted from this column along with only about an equimolar contamination with other lipids (principally PtdCho and PtdEtn), by 200ml of chloroform/methanol (22:3, v/v) containing 0.02% (v/v) conc. HCl.

The eluate was concentrated in vacuo and the lipids spotted on Silica-gel G t.l.c. plates (Merck) pre-sprayed with 1% potassium oxalate. They were developed in chloroform/methanol/water/$NH_3$ (45:35:2:8, by vol.) and the [$^{32}P$] lipids located by autoradiography (2h at room temperature or overnight at $-15°C$). The radioactive inosites were scraped off and eluted with 200ml of chloroform/methanol/water/HCl (145:245:115:1, by vol.). This was converted into two phases by the addition of 0.8vol. of chloroform and 0.14vol. of 0.9% NaCl, and the lower phase washed once with upper phase containing 10mM-CDTA to remove most of the $CaCl_2$. The lower phase was finally dried down and resuspended in chloroform.

$PtdIns(4,5)P_2$ and PtdIns4P prepared in this

way were chromatographically and radiochemically more than 99% pure. Generally one donation of blood incubated with 5–10 mCi of $^{32}$P gave 2–4 $\mu$Ci of radioactive PtdIns(4,5)$P_2$.

### PtdIns(4,5)$P_2$ phosphodiesterase and phosphomonoesterase assay

The radioactive inositide was mixed with PtdIns(4,5)$P_2$ prepared from ox or sheep brain to give 10000 d.p.m. per tube. The final assay volume was 0.3 ml, containing 0.15 ml of Tris/maleate/KOH buffer, final concn. 50 mM, with or without EDTA or EGTA (see below), enzyme and water.

Ca$^{2+}$ and Mg$^{2+}$ concentrations were varied by using Ca$^{2+}$/EDTA or Mg$^{2+}$/EDTA buffers calculated as described by Raaflaub (1956). We also used Ca$^{2+}$/EGTA buffers containing (final concn. in assay) Mg$^{2+}$ (1 or 3 mM) and 80 mM-KCl. All such buffers were made with 6 mM-EGTA or -EDTA, the necessary calcium (or Mg$^{2+}$) additions, plus double-strength Mg$^{2+}$ and KCl for the EGTA buffers, and diluted twice in the final assay. Generally we used 20 nmol of substrate per tube, 3–10 $\mu$l of brain enzyme (see below), and incubations were for 10 min at 37°C. For experiments using pure PtdIns(4,5)$P_2$ as a substrate (Figs. 1 and 2 below), 40 nmol was used. For some other experiments (Fig. 3) the PtdIns(4,5)$P_2$ (20 nmol) was admixed with 200 nmol of a mixture containing (% by weight of phosphorus) PtdIns (24%), PtdEtn (24%), PtdCho (13%), PtdSer (23%), sphingomyelin (16%) plus approx. 25% (weight of total phospholipids) of cholesterol, which is similar in composition to the inner half of the rat liver sinusoidal membrane (Higgins & Evans, 1978; Dawson et al., 1980). For other experiments (Figs. 4 and 5), 20 nmol of PtdIns(4,5)$P_2$ was mixed with 200 nmol of PtdCho or PtdEtn or a mixture of these two. Blanks were either tubes with enzyme added at the end, or assays with EDTA (or EGTA) and no added Ca$^{2+}$ (for phosphodiesterase studies).

### Phosphodiesterase versus monoesterase

After incubation the reaction was stopped by the addition of trichloroacetic acid, then of serum albumin, centrifugation, removal of the acid by four washes with diethyl ether, followed by neutralization with NH$_3$ (Irvine et al., 1977). The possible products of the reaction, P$_i$, Ins(1,4)$P_2$ and Ins(1,4,5)$P_3$, were separated on Dowex (formate form) columns as described by Downes & Michell (1981). In some experiments we de-salted the Ins(1,4,5)$P_3$ fraction by using Dowex (Cl$^-$ form) and LiCl (Downes et al., 1982) and checked by ionophoresis and autoradiography that all the radioactivity was indeed in Ins(1,4,5)$P_3$.

There is a possibility that some of the P$_i$ is formed not by monoesterase action on

PtdIns(4,5)$P_2$, but from phosphatase activity on Ins(1,4,5)$P_3$. We found that the brain supernatant contains soluble phosphatases capable of hydrolysing Ins(1,4,5)$P_3$, so we undertook a series of control experiments using non-radioactive lipids and [$^{32}$P]Ins(1,4,5)$P_3$ and [$^{32}$P]Ins(1,4)$P_2$ prepared from the same erythrocyte ghosts as those used for that particular batch of PtdIns(4,5)$P_2$ and PtdIns4$P$ (and therefore of similar specific radioactivity). [$^{32}$P]Ins(1,4,5)$P_3$ and [$^{32}$P]Ins(1,4)$P_2$ were added to the tubes at similar levels to those that would be released during phosphodiesterase assays. With no Mg$^{2+}$ present there was no significant breakdown of Ins(1,4,5)$P_3$ and Ins(1,4)$P_2$ unless more than 50 $\mu$l of brain enzyme were added or the incubation time was greater than 40 min. With 1 mM-Mg$^{2+}$ there was some breakdown, but this was not significant ($<5\%$) under the conditions employed in the experiments reported here, namely 3–5 $\mu$l of enzyme per tube and 10 min incubations. In some experiments we added an Ins(1,4,5)$P_3$ 'trap', which had no effect on the release of radiolabel from PtdIns(4,5)$P_2$ into any of the products. Thus, under the conditions used here (i.e. provided the enzyme levels are low and the incubation times short), the release of P$_i$ is a true measure of phosphomonoesterase activity towards the lipid, and we can therefore assay monoesterase and phosphodiesterase at the same time.

### Linearity of assay reaction

In preliminary experiments with the phospholipid mixture approximating to liver sinusoidal membranes (see above) we experienced some difficulty in obtaining linearity of the reaction both with time, and with addition of enzyme, under these conditions. Experiments with sequential additions of enzyme or substrate established that, despite sonication of the lipid mixture (to form unilamellar vesicles), only a proportion (20–35% depending on the experiment and conditions) of the substrate was available for hydrolysis. We therefore standardized most of our assays by using only 3–5 $\mu$l of brain supernatant, so that the enzyme reactions of both monoesterase and diesterase are linear with time up to at least 20 min, and 10 min was the usual period of incubation.

### Results

#### pH-dependence

Fig. 1 shows the effect of pH on PtdIns(4,5)$P_2$ phosphodiesterase activity under a variety of conditions. With a pure PtdIns(4,5)$P_2$ substrate we found that Ca$^{2+}$ at concentrations higher than 0.1 mM inhibited the activity (see below), perhaps owing to precipitation of the substrate; so for 1 mM-Ca$^{2+}$ (closed circles), and 1 $\mu$M-Ca$^{2+}$ + 1 mM-Mg$^{2+}$
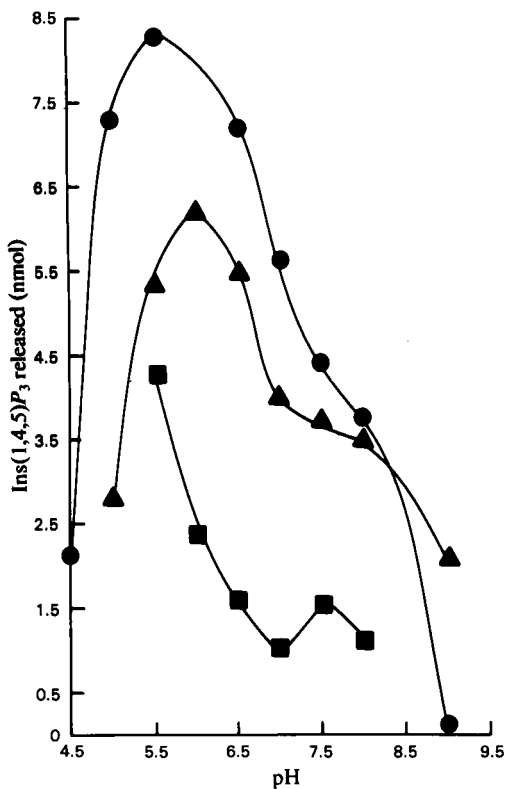
Fig. 1. *Effect of pH on PtdIns(4,5)P₂ phosphodiesterase activity*

PtdIns(4,5)$P_2$ phosphodiesterase was assayed as described in the Materials and methods section under three different ionic conditions: ▲, $Ca^{2+}$/EDTA buffers (free $Ca^{2+}$ 1 $\mu$M); ●, 1 mM-$Ca^{2+}$, 80 mM-KCl; ■, $Ca^{2+}$/EGTA buffers (free $Ca^{2+}$ 1 $\mu$M), 1 mM-$Mg^{2+}$, 80 mM-KCl. For the last buffer series (■) the pH range could not be extended below 5.5 because of the low $Ca^{2+}$-buffering power of EGTA below this pH. The pH was varied in all of these by 50 mM-(final concn.) Tris/maleate/NaOH buffers. All the curves shown are typical of at least two separate experiments for each ionic condition. For the assays with 1 mM-$Ca^{2+}$ or 1 mM-$Mg^{2+}$ present (● and ■), the PtdIns(4,5)$P_2$ was not presented as a pure substrate but was admixed with other phospholipids (see the text).

(closed squares), we employed a lipid mixture approximating to the composition of the inner half of a bilayer of a plasma membrane (see the Materials and methods section), or a fivefold excess of phosphatidylethanolamine (see below) respectively. PtdIns(4,5)$P_2$ was 9% of the total phospholipid in the former mixture, a level probably greater than that found even in a plasma membrane *in vivo*, but one dictated by the specific radioactivity and quantity of $^{32}$P-labelled substrate available. It is apparent from Fig. 1 that the pH profile of the phosphodiesterase is broadly similar

whether the assay mixture contains 1 $\mu$M-$Ca^{2+}$ alone, 1 $\mu$M-$Ca^{2+}$ plus 1 mM-$Mg^{2+}$ + 80 mM-KCl, or 1 mM-$Ca^{2+}$ + 80 mM-KCl. In this respect it differs from the PtdIns phosphodiesterase of rat brain, which shows markedly differing pH optima depending on the charge distribution on the substrate (Irvine *et al.*, 1984). It resembles PtdIns phosphodiesterase, however, in having a main peak of activity at pH approx. 5–5.5 and a minor one at 7.5–8.0. Previous estimates of the pH optimum of PtdIns(4,5)$P_2$ phosphodiesterase have generally been conducted with cetyltrimethylammonium bromide present (see Irvine, 1982, for references), and the data in Fig. 1 are probably a truer reflection of the enzyme(s) pH requirements. As the PtdIns phosphodiesterase activities at pH 5 and 7.5 can be partially resolved into different protein species (Hirasawa *et al.*, 1982), this raises the interesting question of whether PtdIns(4,5)$P_2$ phosphodiesterase shows a similar heterogeneity, and the most important observations made below were tested at two different pH values for that reason.

### Bivalent-cation-dependency

Because of the ability of PtdIns(4,5)$P_2$ to bind bivalent cations, and hence possibly to alter the free-cation concentration of the medium, it is necessary to employ EDTA or EGTA buffers for low free-cation concentrations. Fig. 2 shows the $Ca^{2+}$- and $Mg^{2+}$-dependency of PtdIns(4,5)$P_2$ phosphodiesterase and monoesterase activity of the rat brain supernatant with the pure PtdIns(4,5)$P_2$ substrate. Only by adding 10 mM-CDTA or EDTA could the phosphodiesterase and phosphomonoesterase be completely inhibited, and the probable reason is that there may be traces of $Ca^{2+}$ bound to the substrate; the CDTA wash in the preparation (see the Materials and methods section) will be of limited efficiency in the presence of the HCl at that stage. The monoesterase can use $Ca^{2+}$ (Fig. 2), confirming the observation of Nijjar & Hawthorne (1977), but clearly magnesium is a better activator (Dawson & Thompson, 1964; Nijjar & Hawthorne, 1977; Irvine, 1982). The stimulation of the phosphodiesterase at higher $Mg^{2+}$ concentrations may be caused by displacement of traces of bound $Ca^{2+}$ by the $Mg^{2+}$. The inhibitions observed at high $Ca^{2+}$ concentrations may be caused by substrate precipitation, though $Mg^{2+}$ is different from $Ca^{2+}$ in this respect. In all subsequent experiments (where it was required, for example, to have 1 mM-$Mg^{2+}$ present or to increase $Ca^{2+}$ to millimolar levels), the PtdIns(4,5)$P_2$ substrate was mixed with a tenfold excess of the lipid mixtures mentioned above.

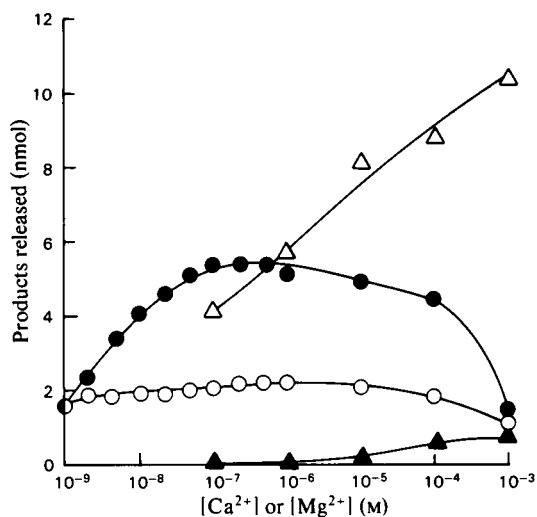Downes & Michell (1982*b*), when studying the membrane-bound PtdIns(4,5)$P_2$ phosphodiester-

Fig. 2. *Effect of $Ca^{2+}$ and $Mg^{2+}$ on $PtdIns(4,5)P_2$ phosphodiesterase and monoesterase activity*
The substrate was the pure phospholipid at 40 nmol per tube, and $Mg^{2+}$ and $Ca^{2+}$ concentrations were varied by EDTA buffers at pH 7.25 (see the Materials and methods section). Above $10^{-4}$ M (100 $\mu$M) the relevant bivalent cation was added in excess of the EDTA to reach the desired concentration. Circles ($\bullet$, $\bigcirc$) represent the effect of $Ca^{2+}$, and triangles ($\blacktriangle$, $\triangle$) that of $Mg^{2+}$. Closed symbols ($\bullet$, $\blacktriangle$), phosphodiesterase activity; open symbols ($\bigcirc$, $\triangle$), phosphomonoesterase activity. The data are the combined results of three experiments conducted on one brain supernatant.



Fig. 3. *Effect of $Ca^{2+}$ on $PtdIns(4,5)P_2$ phosphodiesterase and monoesterase activities with and without $Mg^{2+}$ and KCl*
The substrate was presented as 10% of a complex phospholipid mixture (see the Materials and methods section). The $Ca^{2+}$ concentration was varied using $Ca^{2+}$/EDTA buffers, or $Ca^{2+}$/EGTA buffers with 3 mM-$Mg^{2+}$ and 80 mM-KCl (final concns.) present. The pH was 7.25, and $Ca^{2+}$ was added in excess of EDTA above $10^{-4}$ M (100 $\mu$M). Triangles ($\blacktriangle$, $\triangle$), $Ca^{2+}$/EDTA buffers, no $Mg^{2+}$ or KCl; circles ($\bullet$, $\bigcirc$), $Ca^{2+}$/EGTA buffers + $Mg^{2+}$ + KCl. Open symbols ($\bigcirc$, $\triangle$), phosphomonoesterase activity; closed symbols ($\bullet$, $\blacktriangle$), phosphodiesterase activity. Very similar results were obtained in two separate and identical experiments.

ase of human erythrocytes, made the important observation that raising both the ionic strength and the $Mg^{2+}$ concentration to values nearer to those likely to be found *in vivo*, had a profound effect on the $Ca^{2+}$-dependency of the enzyme. Instead of requiring micromolar levels of $Ca^{2+}$ (as does the enzyme here; Fig. 2), the erythrocyte enzyme needed millimolar levels of $Ca^{2+}$ for activity, and Downes & Michell (1982b) suggested this as a possible reason for the stability of $PtdIns(4,5)P_2$ in red blood cells (i.e. the calcium concentration inside blood cells will never reach the millimolar levels the enzyme needs when $Mg^{2+}$ and $K^+$ are present at physiological concentrations). The data in Fig. 3 show that, if given the $PtdIns(4,5)P_2$ as part of an 'inner-monolayer' mixture, the soluble rat brain $PtdIns(4,5)P_2$ phosphodiesterase behaves in an identical fashion, with little activity below 1 mM-$Ca^{2+}$ if $Mg^{2+}$ and $K^+$ are present.

We explored a number of lipid mixtures to see if any component of the 'inner-monolayer' mixture might be inhibitory under these conditions, because we have found that choline-containing phospholipids are inhibitory for PtdIns phosphodiester-
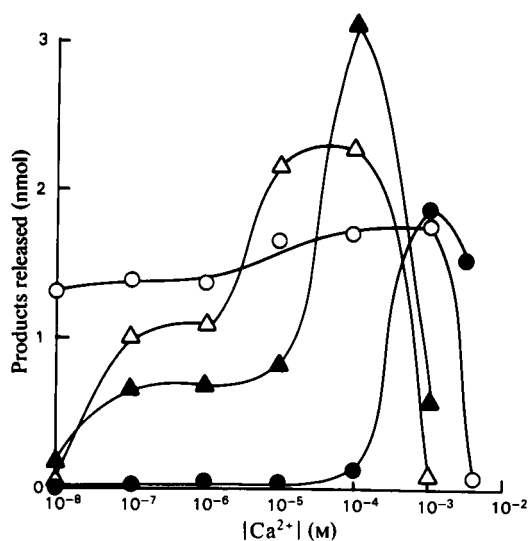
ase (Irvine *et al.*, 1979). Substituting for the phosphatidylcholine with PtdIns had no effect (results not shown). However, replacing PtdCho in the lipid mixture by PtdEtn gave a small activity at 1 $\mu$M-$Ca^{2+}$, 1 mM-$Mg^{2+}$ and 80 mM-KCl, and this suggested the possibility that, under these ionic conditions, the $PtdIns(4,5)P_2$ phosphodiesterase may be able to attack a non-bilayer configuration of a type which PtdEtn induces (Cullis & de Kruiff, 1979; de Kruiff *et al.*, 1980), as has been found obligatory for the phospholipase $A_1$ of rat liver cytosol (Dawson *et al.*, 1983).

Fig. 4($a$) shows the effect of converting the substrate from a bilayer into a non-bilayer transitional configuration by changing the PtdCho/PtdEtn ratio of the lipid mixture in which the $PtdIns(4,5)P_2$ was presented (as 9% of the total lipid). Both monoesterase and diesterase at 1 $\mu$M-$Ca^{2+}$, 1 mM-$Mg^{2+}$ and 80 mM-KCl are stimulated by a change away from the purely bilayer configuration, at pH 7.25 (Fig. 4$a$) and at pH 5.5 (Fig.

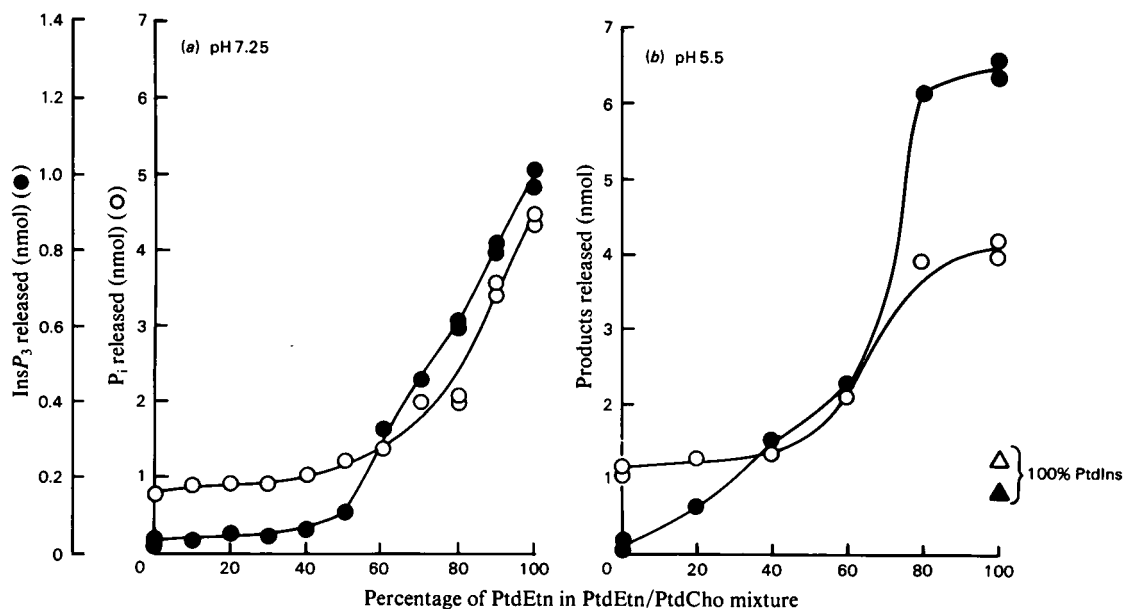R. F. Irvine, A. J. Letcher and R. M. C. Dawson



Fig. 4. *Effect of phosphatidylcholine/phosphatidylethanolamine mixtures on PtdIns(4,5)P₂ phosphodiesterase and phospho-monoesterase activities*

The substrate was presented as 20 nmol admixed with 200 nmol of a PtdEtn/PtdCho mixture of various proportions. The assay was at 1 $\mu$M-free $Ca^{2+}$ ($Ca^{2+}$/EGTA buffer), 1 mM-$Mg^{2+}$, 80 mM-KCl and (*a*) pH 7.25 or (*b*) pH 5.5. Open symbols ($\bigcirc$, $\triangle$), phosphomonoesterase activity; closed symbols ($\bullet$, $\blacktriangle$), phosphodiesterase activity. The data are the combined results of three separate experiments, though the results represented by triangles ($\triangle$, $\blacktriangle$) are derived from one experiment in which PtdIns(4,5)P₂ was admixed with PtdIns instead of the PtdCho/PtdEtn mixture.

4*b*). In both instances the effect on the diesterase was the greater, especially at the lower pH (Fig. 4*b*), and it is possible that the monoesterase stimulation can be mostly accounted for by greater substrate availability (see the Materials and methods section under 'Linearity of assay reaction'). This marked stimulation of PtdIns(4,5)P₂ phosphodiesterase was identical at 100 nM-$Ca^{2+}$ (results not shown), and the $Ca^{2+}$-dependence of the activity with $Mg^{2+}$ and KCl present when PtdEtn is admixed with the substrate (Fig. 5) confirms the ability of the enzyme to attack its substrate under an approximate physiological ionic environment if the substrate is presented in this form. Finally, we also found that PtdIns(4,5)P₂ phosphodiesterase could hydrolyse its substrate in the 'inner-mono-layer' lipid mixture if for every mol of lipid we added 0.4 mol of 1-stearoyl-2-arachidonoylglycerol (prepared from pig liver PtdIns), possibly by inducing a departure from the close-packed ordered bilayer structure (results not shown). This supports the suggestion that it is the configuration of the lipids, rather than their chemical nature, which is important for activity. Nevertheless, we can only infer from these experiments that the controlling factor on PtdIns(4,5)P₂ phosphodiesterase activity is the lipid configuration, and

further experiments using other phospholipids will be necessary to establish this with a greater degree of certainty.

*Relative activity of PtdIns(4,5)P₂ phosphodiesterase and PtdIns phosphodiesterase*

The determination of whether a phospholipase is specific for its substrate is often difficult (Dawson, 1973; Irvine, 1982). It is not certain, for example, whether PtdIns phosphodiesterase and PtdIns(4,5)P₂ phosphodiesterase are distinct entities, because the data here and elsewhere (Irvine *et al.*, 1984) show that both activities are heterogeneous and are very particular about the conditions of their environment and the presentation of their substrate. Recent suggestions that the initial receptor-linked event in many tissues is not a stimulation of PtdIns phosphodiesterase, but rather of PtdIns(4,5)P₂ phosphodiesterase (Michell *et al.*, 1981; Berridge, 1983; Berridge *et al.*, 1983) leads to the question of whether PtdIns phosphodiesterase exists as a distinct (and specific) protein. Low & Weglicki (1983) in a detailed study on myocardial PtdIns and PtdIns(4,5)P₂ phosphodiesterases were unable to separate the two activities. Whether or not they are separate enzymes, if poly-phosphoinositides are the first inositides cata-
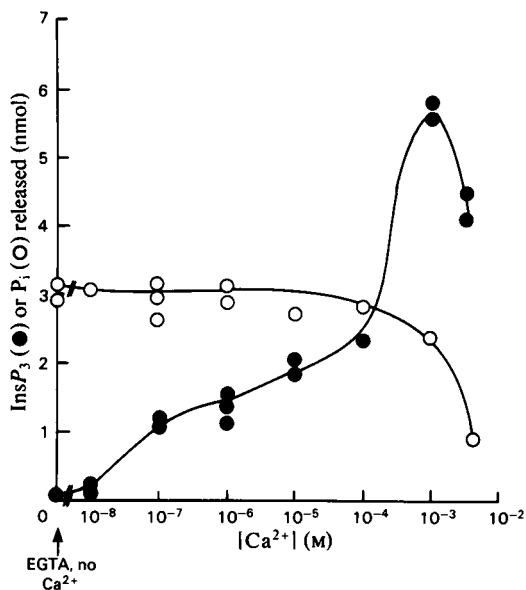
Fig. 5. *Effect of $Ca^{2+}$ on PtdIns(4,5)$P_2$ phosphodiesterase and phosphomonoesterase activity with a non-bilayer substrate*

The substrate was presented admixed with a tenfold excess of PtdEtn. The $Ca^{2+}$ concentration was varied by using $Ca^{2+}$/EGTA buffers with 3 mM-$Mg^{2+}$ and 80 mM-KCl present. The pH of the assay was 7.25. ○, Phosphomonoesterase activity; ●, phosphodiesterase activity. The data are the combined results of three separate experiments.

bolized in stimulated cells, then the relative level of PtdIns(4,5)$P_2$ phosphodiesterase and PtdIns phosphodiesterase in the same rat brain supernatant is of interest. We have therefore compared these two activities at 1 $\mu$M-$Ca^{2+}$, 1 mM-$Mg^{2+}$, 80 mM-KCl and pH 7.25 or 5.5, by using a 'non bilayer' lipid mixture (i.e. PtdEtn in excess), which may induce a substrate presentation similar to that caused by receptor activation (discussed below). If PtdIns(4,5)$P_2$ and PtdIns phosphodiesterases are assayed as here, i.e. 20 nmol of substrate (+200 nmol of PtdEtn), then at pH 7.25 the activities were 251 nmol/h per mg of protein for PtdIns(4,5)$P_2$ and 2.0 nmol/h per mg of protein for PtdIns (all results are means of duplicates with less than 10% variation, and are typical of results obtained from two separate experiments). At pH 5.5 the corresponding values were 1137 and 14 respectively.

*In vivo*, PtdIns is in excess of PtdIns(4,5)$P_2$ and PtdIns4$P$; the degree of this excess depends on the subcellular location, so for a rough approximation we employed a mixture (per tube) of 20 nmol of PtdIns(4,5)$P_2$, 200 nmol of PtdIns and 1000 nmol of PtdEtn, with either the PtdIns(4,5)$P_2$ or the

PtdIns radiolabelled. The activities of PtdIns phosphodiesterase were 18 nmol and 80 nmol/h per mg of protein at pH 7.25 and 5.5 (1 $\mu$M free $Ca^{2+}$, 1 mM-$Mg^{2+}$, 80 mM-KCl) respectively. The corresponding values for PtdIns(4,5)$P_2$ under these conditions were 140 and 485 nmol/h per mg of protein respectively.

### PtdIns4P phosphodiesterase and monoesterase

The method used to prepare [$^{32}$P]PtdIns(4,5)$P_2$ here gave us very small yields of PtdIns4$P$, at a much lower (5–10-fold) specific radioactivity, so only a few experiments could be performed. We had not sufficient radioactivity to repeat the studies using the substrate in an 'inner-monolayer' lipid mixture, but we did study the $Ca^{2+}$-dependency, $Mg^{2+}$-dependency, effect of other lipids, pH-dependence and effect of enzyme concentration on the PtdIns4$P$ phosphodiesterase as compared with the PtdIns(4,5)$P_2$ phosphodiesterase using the pure substrate at a low concentration; the behaviour of the two activities was virtually identical. Several authors have argued that the same enzyme(s) attack both substrates (Hawthorne & White, 1975; Downes & Michell, 1981; Irvine, 1982), and it seems likely that the properties of PtdIns(4,5)$P_2$ phosphodiesterase studied here could be extended to PtdIns4$P$ phosphodiesterase.

### Discussion

Our previous studies on brain PtdIns phosphodiesterase have demonstrated possible control mechanisms and have also emphasized the need to consider carefully the ionic environment under which the enzyme is assayed (Irvine *et al.*, 1979; Dawson *et al.*, 1980; Hirasawa *et al.*, 1981; Irvine *et al.*, 1984). To our knowledge, the brain PtdIns(4,5)$P_2$ phosphodiesterase has not yet been explored in a similar fashion, and the results here represent an initial stage in such a study and give a preliminary insight into what conditions the PtdIns(4,5)$P_2$ phosphodiesterase (and to a lesser extent, the monoesterase) requires.

It seems likely that, during cell stimulation, the catabolism of PtdIns(4,5)$P_2$ occurs in the plasma membrane (Michell, 1975; Downes & Michell, 1982a). PtdIns(4,5)$P_2$ might be either uniformly distributed as a minor constituent of the lipid bilayer, or alternatively collected in specific regions (in or near receptor sites). In the first instance, although the polyphosphoinositide itself is extremely acidic, possessing five fully ionized charges at a physiological pH (Hauser & Dawson, 1967), the disruptive effect of this on the bilayer structure is likely to be modified by the counterion-binding properties of cations within the cell, particularly $Mg^{2+}$. With regard to an association of

PtdIns(4,5)$P_2$ with receptors, it is likely that this phospholipid will have an affinity for the more basic proteins within the plasma membrane; there is indirect evidence that the comparatively high content of polyphosphoinositide in myelin (which is an extension of the oligodendrial plasma membrane) is associated with the myelin basic protein (Palmer & Dawson, 1969).

There is no doubting the potential of the rat brain supernatant phosphodiesterase to hydrolyse PtdIns(4,5)$P_2$, but, as with PtdIns phosphodiesterase, it is probably inhibited from doing so *in vivo* by the state of its substrate in the membrane environment. Although the enzyme will hydrolyse PtdIns(4,5)$P_2$ in any form at $1 \mu M$-$Ca^{2+}$ (Figs. 2 and 3), if $Mg^{2+}$ (1 mM) and KCl (80 mM) are present, then it cannot attack PtdIns(4,5)$P_2$ when this is part of a lipid mixture similar to that in which it may exist in the inner-bilayer lamella *in vivo* (Fig. 3). Perturbation of that lipid structure by mixing the substrate with an excess of PtdEtn (Fig. 4) enables the enzyme to hydrolyse its substrate, and we suggest that this perturbation may be analogous to that which occurs in plasma-membrane structure after receptor activation.

The $Ca^{2+}$-dependency of PtdIns(4,5)$P_2$ phosphodiesterase hydrolysing a non-bilayer substrate (Fig. 5) shows that, like PtdIns phosphodiesterase under similar conditions (Irvine *et al.*, 1984), changes in $Ca^{2+}$ within likely physiological limits (100 nm–10 $\mu M$) have little effect on the activity. The low activity at 10 nm-free $Ca^{2+}$ (Fig. 5) may be an artefact of the low level of total $Ca^{2+}$ added to make the 10 nM free-$Ca^{2+}$/EGTA buffer; for example, the substrate could compete with EGTA for the low levels of $Ca^{2+}$ available, so that the free level is actually even lower. If however, the enzyme does truly show a sharp cut-off below 100 nM-$Ca^{2+}$ (Fig. 5), this may explain why, in some people's hands, EGTA in the external medium can abolish the PtdIns(4,5)$P_2$ hydrolysis stimulated by agonists (Prpić *et al.*, 1983), whereas in others (even using the same tissue) it does not (Creba *et al.*, 1983), because the actual free-calcium level in a tissue incubated in EGTA may depend precisely on the state of the internal stores, permeability of the plasma membrane, etc.

In the present study we have used soluble enzyme activities because of the ease of assaying and manipulating them. Whether or not the receptor-activated enzyme is soluble or membrane-bound is a contentious point (Irvine, 1982); it may even be a matter of semantics: to hydrolyse a membrane-bound substrate the soluble activity studied here must bind to the lipid phase. The similarity in the behaviour of the $Ca^{2+}$-dependency (with or without $Mg^{2+}$ and KCl; Fig. 3) with that shown by the membrane-bound PtdIns(4,5)$P_2$ phosphodi-

esterase of human erythrocyte ghosts (Downes & Michell, 1982b) at least encourages us to think that soluble or membrane-bound enzymes may behave similarly as regards their ionic requirements for activity. If that is so, then, in this instance, binding of the enzyme to the substrate may not be a rate-limiting step, as Pieroni & Verger (1983) have also recently shown for porcine phospholipase $A_2$ hydrolysing a phospholipid/triacylglycerol mixture. If a membrane-bound PtdIns(4,5)$P_2$ phosphodiesterase exists *in vivo*, then it seems likely that it too could be activated, on similar principles, to the soluble activities studied here.

In conclusion, the results here suggest a concept of PtdIns(4,5)$P_2$ phosphodiesterase which has sufficient calcium *in vivo* to hydrolyse its substrate, but where the physicochemical form in which the substrate exists in a non-stimulated plasma membrane is unsuitable for hydrolysis. In contrast, the monoesterase(s), according to our present data, could probably hydrolyse PtdIns(4,5)$P$ contained in this same substrate structure, thus accounting for the high rate of turnover of the 4'- and 5'-phosphate groups in resting tissues. A perturbation of membrane structure, which could be localized and rapid, and concomitantly cause the activity of the phosphodiesterase to rise to a level comparable with (or greater than) that of the monoesterase (Fig. 4), is consistent with the rapid and dramatic increase in phosphodiesterasic polyphosphoinositide hydrolysis observed in, for example, liver (Creba *et al.*, 1983) or blowfly salivary gland (Berridge, 1983). Similar principles, but on a reduced scale (i.e. smaller, and perhaps more stable, changes in plasma-membrane structure) may also account for the slower and less pronounced increases in inositide turnover which accompany cell proliferation (Diringer & Friis, 1977; Michell, 1982).

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