Biochem. J. (1984) **218**, 177–185
Printed in Great Britain

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

Some properties and possible control mechanisms

Robin F. IRVINE, Andrew J. LETCHER and Rex M. C. DAWSON
Department of Biochemistry, ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

(*Received 15 August 1983/Accepted 21 October 1983*)

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 100nM-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 3mM-Mg^{2+} and 80mM-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 1mM-Ca^{2+}. The monoesterase can hydrolyse PtdIns(4,5)P_2 in such a lipid mixture at all Ca^{2+} concentrations with 1 or 3mM-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+}; 1mM-Mg^{2+}; 80mM-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 1mM-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 pH7.25 (or 5.5), 1 μM-Ca^{2+}, 1mM-Mg^{2+} and 80mM-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, 1982*a*; Abdel-Latif, 1983), and recent evidence suggests that the primary stimulated pathway is by phosphodiesterase action on PtdIns(4,5)P_2 and PtdIns4*P*, 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. PtdIns4*P* 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 (1982*b*) 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 (500*g*), 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 inositides 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 PtdIns4*P* 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 inositides 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 PtdIns4*P* prepared in this

way were chromatographically and radiochemically more than 99% pure. Generally one donation of blood incubated with 5–10mCi of ^{32}P gave 2–4μCi 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 10000d.p.m. per tube. The final assay volume was 0.3ml, containing 0.15ml of Tris/maleate/KOH buffer, final concn. 50mM, 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 3mM) and 80mM-KCl. All such buffers were made with 6mM-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 20nmol of substrate per tube, 3–10μl of brain enzyme (see below), and incubations were for 10min at 37°C. For experiments using pure PtdIns(4,5)P_2 as a substrate (Figs. 1 and 2 below), 40nmol was used. For some other experiments (Fig. 3) the PtdIns(4,5)P_2 (20nmol) was admixed with 200nmol 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), 20nmol of PtdIns(4,5)P_2 was mixed with 200nmol 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 PtdIns4P (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 50μl of brain enzyme were added or the incubation time was greater than 40min. With 1mM-Mg^{2+} there was some breakdown, but this was not significant (<5%) under the conditions employed in the experiments reported here, namely 3–5μl of enzyme per tube and 10min 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 3–5μl of brain supernatant, so that the enzyme reactions of both monoesterase and diesterase are linear with time up to at least 20min, and 10min 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 0.1mM inhibited the activity (see below), perhaps owing to precipitation of the substrate; so for 1mM-Ca^{2+} (closed circles), and 1μM-Ca^{2+} + 1mM-Mg^{2+}

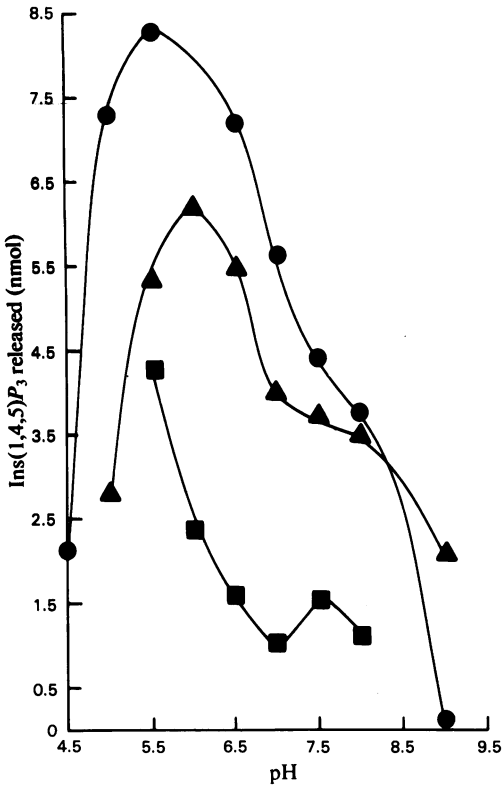

Fig. 1. *Effect of pH on PtdIns(4,5)P_2 phosphodiesterase activity*

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 μM); ●, 1mM-Ca^{2+}, 80mM-KCl; ■, Ca^{2+}/EGTA buffers (free Ca^{2+} 1 μM), 1mM-Mg^{2+}, 80mM-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 50mM-(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 1mM-Ca^{2+} or 1mM-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 μM-Ca^{2+} alone, 1 μM-Ca^{2+} plus 1mM-Mg^{2+} + 80mM-KCl, or 1mM-Ca^{2+} + 80mM-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 10mM-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 1mM-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-

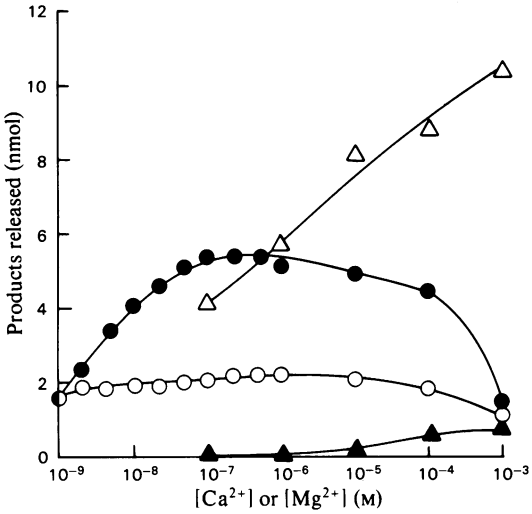

Fig. 2. *Effect of Ca^{2+} and Mg^{2+} on PtdIns(4,5)P_2 phosphodiesterase and monoesterase activity*

The substrate was the pure phospholipid at 40nmol per tube, and Mg^{2+} and Ca^{2+} concentrations were varied by EDTA buffers at pH7.25 (see the Materials and methods section). Above 10^{-4}M (100μM) the relevant bivalent cation was added in excess of the EDTA to reach the desired concentration. Circles (●, ○) represent the effect of Ca^{2+}, and triangles (▲, △) that of Mg^{2+}. Closed symbols (●, ▲), phosphodiesterase activity; open symbols (○, △), phosphomonoesterase activity. The data are the combined results of three experiments conducted on one brain supernatant.

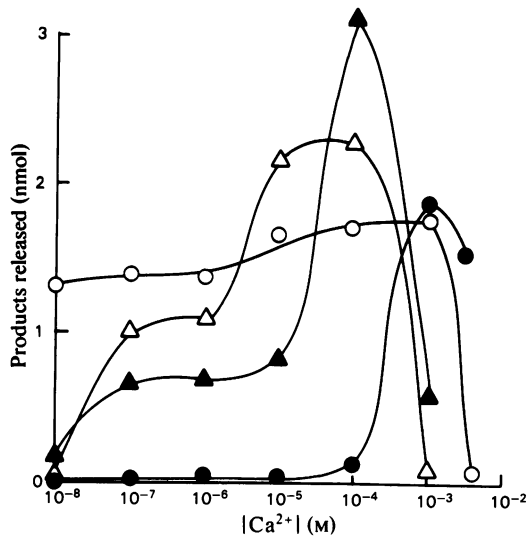

Fig. 3. *Effect of Ca^{2+} on PtdIns(4,5)P_2 phosphodiesterase and monoesterase activities with and without Mg^{2+} and KCl*

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 3mM-Mg^{2+} and 80mM-KCl (final concns.) present. The pH was 7.25, and Ca^{2+} was added in excess of EDTA above 10^{-4}M (100μM). Triangles (▲, △), Ca^{2+}/EDTA buffers, no Mg^{2+} or KCl; circles (●, ○), Ca^{2+}/EGTA buffers + Mg^{2+} + KCl. Open symbols (○, △), phosphomonoesterase activity; closed symbols (●, ▲), 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 (1982*b*) 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 1mM-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 phosphodiesterase (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 1μM-Ca^{2+}, 1mM-Mg^{2+} and 80mM-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 1μM-Ca^{2+}, 1mM-Mg^{2+} and 80mM-KCl are stimulated by a change away from the purely bilayer configuration, at pH7.25 (Fig. 4*a*) and at pH5.5 (Fig.

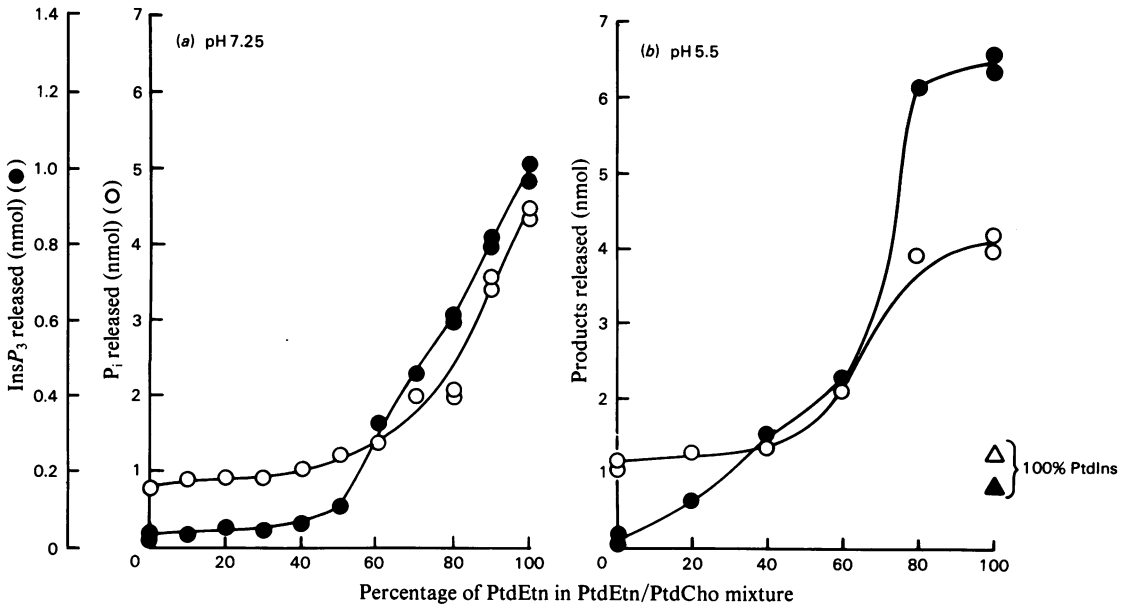

Fig. 4. *Effect of phosphatidylcholine/phosphatidylethanolamine mixtures on PtdIns(4,5)P_2 phosphodiesterase and phosphomonoesterase activities*

The substrate was presented as 20nmol admixed with 200nmol of a PtdEtn/PtdCho mixture of various proportions. The assay was at 1 μM-free Ca^{2+} (Ca^{2+}/EGTA buffer), 1mM-Mg^{2+}, 80mM-KCl and (*a*) pH7.25 or (*b*) pH5.5. Open symbols (○, △), phosphomonoesterase activity; closed symbols (●, ▲), phosphodiesterase activity. The data are the combined results of three separate experiments, though the results represented by triangles (△, ▲) are derived from one experiment in which PtdIns(4,5)P_2 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)P_2 phosphodiesterase was identical at 100nM-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)P_2 phosphodiesterase could hydrolyse its substrate in the 'inner-monolayer' lipid mixture if for every mol of lipid we added 0.4mol 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)P_2 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)P_2 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)P_2 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)P_2 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)P_2 phosphodiesterases were unable to separate the two activities. Whether or not they are separate enzymes, if polyphosphoinositides are the first inositides cata-

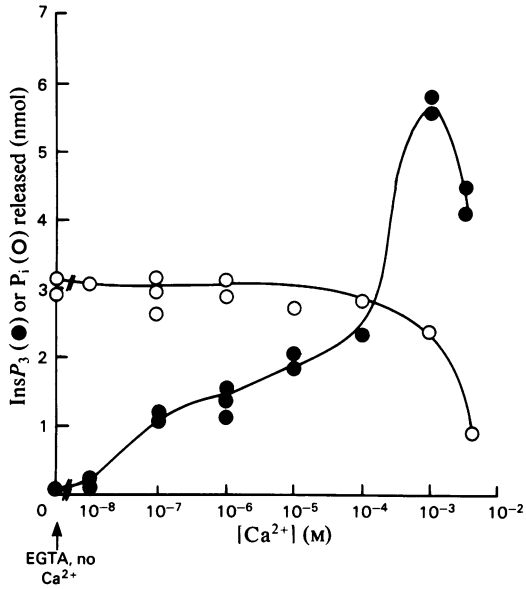

Fig. 5. *Effect of Ca^{2+} on PtdIns(4,5)P_2 phosphodiesterase and phosphomonoesterase activity with a non-bilayer substrate*

The substrate was presented admixed with a tenfold excess of PtdEtn. The Ca^{2+} concentration was varied by using Ca^{2+}/EGTA buffers with 3mM-Mg^{2+} and 80mM-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 μM-Ca^{2+}, 1mM-Mg^{2+}, 80mM-KCl and pH7.25 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. 20nmol of substrate (+200nmol of PtdEtn), then at pH7.25 the activities were 251nmol/h per mg of protein for PtdIns(4,5)P_2 and 2.0nmol/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 pH5.5 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 20nmol of PtdIns(4,5)P_2, 200nmol of PtdIns and 1000nmol of PtdEtn, with either the PtdIns(4,5)P_2 or the PtdIns radiolabelled. The activities of PtdIns phosphodiesterase were 18nmol and 80nmol/h per mg of protein at pH7.25 and 5.5 (1 μM free Ca^{2+}, 1mM-Mg^{2+}, 80mM-KCl) respectively. The corresponding values for PtdIns(4,5)P_2 under these conditions were 140 and 485nmol/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, 1982*a*). 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 μM-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 μM) 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 phosphodiesterase of human erythrocyte ghosts (Downes & Michell, 1982*b*) 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).

References

Abdel-Latif, A. A. (1983) *Handb. Neurochem.* **3**, 91–131

Agranoff, B., Murthy, P. & Seguin, E. B. (1983) *J. Biol. Chem.* **258**, 2076–2078

Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858

Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482

Creba, J. A., Downes, C. P., Hawkins, P. J., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747

Cullis, P. R. & de Kruiff, B. (1979) *Biochim. Biophys. Acta* **559**, 399–420

Dawson, R. M. C. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 97–116, Elsevier, Amsterdam, London and New York

Dawson, R. M. C. & Thompson, W. (1964) *Biochem. J.* **91**, 244–250

Dawson, R. M. C., Hemington, N. & Irvine, R. F. (1980) *Eur. J. Biochem.* **112**, 33–38
Dawson, R. M. C., Irvine, R. F., Hemington, N. L. & Hirasawa, K. (1983) *Biochem. J.* **209**, 865–872
de Kruiff, B., Rietveld, A. & Cullis, P. R. (1980) *Biochim. Biophys. Acta* **600**, 343–357
Diringer, H. & Friis, R. R. (1977) *Cancer Res.* **37**, 2978–2984
Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
Downes, C. P. & Michell, R. H. (1982*a*) *Cell Calcium* **3**, 467–502
Downes, C. P. & Michell, R.H. (1982*b*) *Biochem. J.* **202**, 53–58
Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177
Hauser, H. & Dawson, R. M. C. (1967) *Eur. J. Biochem.* **1**, 61–69
Hawthorne, J. N. & White, D. A. (1975) *Vitam. Horm. (N.Y.)* **33**, 529–573
Higgins, J. A. & Evans, W. H. (1978) *Biochem. J.* **174**, 563–567
Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1981) *Biochem. J.* **193**, 607–614
Hirasawa, K., Irvine, R. F. & Dawson, R. M. C. (1982) *Biochem. J.* **205**, 437–442
Irvine, R. F. (1982) *Cell Calcium* **3**, 295–309
Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1977) *Biochem. J.* **164**, 277–280
Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1978) *Biochem. J.* **176**, 475–484
Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1979) *Eur. J. Biochem.* **99**, 525–530
Irvine, R. F., Letcher, A. J. & Dawson, R. M. C. (1984) in *Physiological Role of Phospholipids in the Nervous System* (Horrocks, L. A., Kanfer, J. N. & Porcellati, G. A., eds.), Raven Press, New York, in the press
Kaibuchi, K., Sano, K., Hoshijima, M., Takai, Y. & Nishizuka, Y. (1982) *Cell Calcium* **3**, 323–335
Low, M. G. & Weglicki, W. B. (1983) *Biochem. J.* **215**, 325–334
Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
Michell, R. H. (1982) *Cell Calcium* **3**, 429–440
Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 123–137
Nijjar, M. S. & Hawthorne, J. N. (1977) *Biochim. Biophys. Acta* **480**, 390–402
Palmer, F. B. & Dawson, R. M. C. (1969) *Biochem. J.* **111**, 637–646
Pieroni, G. & Verger, R. (1983) *Eur. J. Biochem.* **132**, 639–644
Prpić, V., Blackmore, P. F. & Exton, J. H. (1983) *J. Biol. Chem.* **257**, 11323–11331
Raaflaub, J. (1956) *Methods Biochem. Anal.* **3**, 301–305
Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69