Phosphatidylinositol phosphodiesterase in higher plants

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1. The lower regions of the stem of celery (*Apium graveolens* L.) contain a soluble enzyme that hydrolyses phosphatidylinositol. 2. The lipoidal product of hydrolysis is diacylglycerol, and the water-soluble products are 1:2-cyclic phosphoinositol and phosphoinositol in the approximate proportions of 60% and 40% respectively: this indicates that a phosphodiesterase (phospholipase C-like) activity is cleaving the phosphatidylinositol. 3. The enzyme requires a bivalent cation, Ca^{2+} being the most effective activator. 4. The enzyme has a pH optimum, depending on conditions of assay, of pH 5.9–6.6 and in this pH range shows no detectable activity against phosphatidylcholine or phosphatidylethanolamine. 5. The activity is stimulated by phosphatidic acid and slightly inhibited (30% at concentrations equimolar with phosphatidylinositol) by phosphatidylcholine. 6. The phosphodiesterase was also detected (but not quantified) in the tips of the flowers of cauliflowers, in outer leaves of onion and in the elongating stem of daffodils. 7. The enzyme's properties are compared with equivalent mammalian enzymes, and its possible role in the catabolism of phosphatidylinositol in higher plants is discussed.

The widespread phenomenon in animals of stimulated phosphatidylinositol turnover (Hokin, 1968; Michell, 1975; Fain & Berridge, 1979) has caused considerable interest in the enzymes that degrade this phospholipid and their possible control mechanisms. It appears that there are at least two phosphodiesterases in animals that are specific for phosphatidylinositol, one lysosomal and Ca^{2+} -independent (Irvine *et al.*, 1978; Richards *et al.*, 1979), and the other cytosolic and Ca^{2+} -dependent (Dawson, 1959; Kemp *et al.*, 1961).

To our knowledge, there is as yet no report of a stimulated turnover of phosphatidylinositol in higher plants in response to any 'agonist': changes in overall metabolism of glycerophospholipids in response to hormones or developmental changes do occur (see, for example, Beutelman & Kende, 1977; Irvine & Osborne, 1978), but the agonist-stimulated turnover in mammalian systems is usually highly specific for phosphatidylinositol (Michell, 1975). It is therefore of interest that we report in the present paper the existence of a phosphatidylinositol-specific phosphodiesterase in higher plants. Essentially it is similar to the cytosolic Ca^{2+} -dependent mammalian enzyme mentioned above, but several of its properties (studied with celery as a source) are different from that enzyme; the significance of these properties of the plant enzyme and of its very

existence must await further studies on plant lipid metabolism.

Materials and methods

^{32}P -labelled phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine were prepared from Baker's yeast as described by Irvine *et al.* (1978). The release of water-soluble radioactivity from these substrates (final concentration $50\text{ }\mu\text{M}$) was routinely assayed as described by Irvine *et al.* (1978). Incubations, unless stated otherwise, were for 15 min (continuous shaking) at room temperature, in 100 mM-Tris/maleate buffer at pH 6.6. For identification of water-soluble products, the reaction was stopped by the addition of methanol and chloroform rather than trichloroacetic acid (to prevent hydrolysis of 1:2-cyclic phosphoinositol; Dawson *et al.*, 1971); the products were separated by electrophoresis (Dawson & Clarke, 1972).

Lipoidal products were separated by t.l.c. (Richards *et al.*, 1979). Plant extracts were prepared by centrifuging a 50% homogenate (formed by grinding the tissue in a pestle and mortar with sand and water) for 60 min at $100\,000\text{ g}_{\text{av}}$ and using the resultant supernatant. Phospholipase D was assayed by liberation of water-soluble radioactivity from phosphatidyl[^{14}C]choline (prepared from Bakers'

yeast) at pH 5.0 (acetate buffer) with 30% diethyl ether (Quarles & Dawson, 1969b).

Results and discussion

Products of hydrolysis

The phosphodiesterase activity was first detected during preliminary investigations of the effect of phospholipase D on phosphatidylinositol. This latter enzyme (obtained from Sigma Chemical Co.) will slowly hydrolyse phosphatidylinositol at pH 7.5 to form phosphatidic acid (R. F. Irvine & R. M. C. Dawson, unpublished work). A soluble extract of celery (*Apium graveolens* L.), however, formed diacylglycerol as a lipoidal product almost exclusively; no phosphatidic acid could be detected and we found no evidence for a suitably active phosphatidate phosphohydrolase. Examination of the water-soluble phosphate esters produced by the hydrolysis of [32 P]phosphatidylinositol (see the Materials and methods section) gave the results shown in Table 1. The results show that the products are very similar to those formed from phosphatidylinositol by the soluble Ca^{2+} -dependent mammalian phosphatidylinositol phosphodiesterase (Dawson *et al.*, 1971). The radioactivity in glycerophosphoinositol may, however, be at least, in part, caused by an incomplete separation of this ester from phosphoinositol. Indeed, subsequent separations by a new ionophoretic technique (N. Clarke, unpublished work) showed that there was no label in glycerophosphoinositol. Autoradiography of these separations clearly showed discrete radioactive spots coincident with marker compounds (1:2-cyclic phosphoinositol and phosphoinositol). Addition to the incubation of 0.5 mM-1:2-cyclic phosphoinositol, phosphoinositol or glycerophosphoinositol 'traps', none of which were appreciably hydrolysed during the incubation, did not significantly alter the proportions of any radioactive products formed; this suggests that the products are formed directly from phosphatidylinositol rather than from one another.

Stoichiometry of diacylglycerol release with 1:2-cyclic phosphoinositol + phosphoinositol was not quantified, but diacylglycerol was the only detectable

lipoidal product, and negligible amounts of monoacylglycerol and non-esterified fatty acid were formed, even after incubation overnight.

Cation requirements

Incubations were normally performed with no cation additions. As the celery extract was undialysed, presumably sufficient cations were present in it (the extract was at a dilution of 1 in 12 in the final incubation). Addition of EDTA, or dialysis of the celery extract against water, completely inhibited the activity. Extensive exploration of the concentrations of several cations able to restore activity was not attempted. Calcium, manganese, magnesium and zinc ions, in decreasing order of effectiveness, all partly restored the activity of the dialysed enzyme at a concentration of 2 mM. In each case 4 mM cation was less effective than 2 mM. The ionic requirements of the enzyme therefore seem similar to the lymphocyte phosphatidylinositol phosphodiesterase studied by Allan & Michell (1974) in that Ca^{2+} was the most effective activator. The lymphocyte enzyme has a very low (10 μM at pH 7.0) Ca^{2+} requirement (Allan & Michell, 1974); we have not explored this aspect of the celery enzyme further because it may, as with the mammalian enzyme (Allan & Michell, 1974; Irvine & Dawson, 1980), depend largely on the conditions of assay, so the physiological relevance of the exact concentration of Ca^{2+} required would be doubtful.

pH optimum

Fig. 1 shows the effect of pH on the celery phosphodiesterase. The pH optimum of the lymphocyte phosphatidylinositol phosphodiesterase has been reported to be dependent on the conditions of assay (Allan & Michell, 1974). The conditions for Fig. 1 were a substrate concentration of 50 μM and no added cations; the enzyme shows a broad pH optimum around 6.5. Addition of 7.5 mM- Ca^{2+} had no effect on this pH optimum, but assaying the enzyme with phosphatidylinositol at 2 mM and Ca^{2+} at 1 mM [identical conditions with those used for the brain phosphodiesterase by Irvine & Dawson (1978)] shifted the optimum to pH 5.9. It must be emphasized, however, that as with the exact Ca^{2+}

Table 1. *Products of [32 P]phosphatidylinositol hydrolysis by celery extract*

Assay conditions and separation of water-soluble products were as given in the Materials and methods section. Results of one experiment (done in quadruplicate) are given. Similar results were obtained in a completely separate experiment.

Product	P _i	1:2-Cyclic phosphoinositol	Phosphoinositol	Glycerophosphoinositol
Amount produced (nmol)	0.03 $\pm$ 0.019	1.56 $\pm$ 0.095	0.93 $\pm$ 0.17	0.19 $\pm$ 0.11

Fig. 1. Effect of pH on celery phosphatidylinositol phosphodiesterase

The pH was varied by using a succession of 25 mM-Tris/maleate/NaOH buffers. Assay of release of water-soluble radioactivity from [32 P]-phosphatidylinositol was as described in the Materials and methods section. Blank tubes (enzyme added at the end) were included for all pH values. Results are plotted as nmol of phosphorus released/min against pH. Similar results were obtained in an identical experiment.

requirements (see above) such properties of phosphatidylinositol phosphodiesterase using a pure substrate are of doubtful physiological significance. Suffice to say that parallel assays with the enzyme from rat brain under the above conditions always showed a pH optimum at least one unit more acidic than that of the celery enzyme, demonstrating a difference between the mammalian and plant enzymes.

Separation from phospholipase D, and specificity

Phospholipase D is usually the plant enzyme most active against phosphatidylcholine, and celery is a rich source of this enzyme (Quarles & Dawson, 1969a). Phospholipase D is a phosphodiesterase itself, so it is important to separate the present phosphatidylinositol phosphodiesterase from phospholipase D to ensure that the former activity is not just an unusual mode of attack on phosphatidylinositol by the latter; also the high activity of phospholipase D against, for example, phosphatidylcholine makes studies of the specificity of the phosphatidylinositol phosphodiesterase difficult.

The celery extract was submitted to a preliminary fractionation by precipitation with $(\text{NH}_4)_2\text{SO}_4$. Most of the activity precipitated between 40% and 60% saturation, but an overall loss of activity during the fractionation meant that little enrichment of the specific activity of the enzyme occurred. However,

this precipitation and subsequent dialysis against water completely and reproducibly inactivated phospholipase D, so the identity of the two enzymes seems unlikely. Subsequently we found that storage of the celery extract at -20°C after dialysis against water completely removed any detectable phospholipase D activity. We therefore used such a preparation, still highly active against phosphatidylinositol, to assess activity against [32 P]phosphatidylcholine and [32 P]phosphatidylethanolamine prepared from the same yeast source as phosphatidylinositol. No release of water-soluble radioactivity was detected from phosphatidylcholine or phosphatidylethanolamine at pH 6.5; thus the hydrolysis of these two phospholipids by the phosphodiesterase takes place at less than 0.1% the rate of hydrolysis of phosphatidylinositol under identical conditions.

Effect of other phospholipids

We have shown that the cytosolic Ca^{2+} -dependent mammalian phosphatidylinositol phosphodiesterase is strongly inhibited by choline-containing phospholipids, and markedly stimulated by small amounts of acidic phospholipids, for example, phosphatidic acid (Irvine *et al.*, 1979b). In contrast, the other mammalian phosphatidylinositol phosphodiesterase, the Ca^{2+} -independent enzyme found in lysosomes (Irvine *et al.*, 1978), is unaffected by phosphatidylcholine and inhibited by phosphatidic acid (Irvine & Dawson, 1980). The data in Fig. 2 show the results of similar experiments to determine

Fig. 2. Effect of phosphatidylcholine on celery phosphatidylinositol phosphodiesterase

The assay of release of water-soluble radioactivity from [32 P]phosphatidylinositol was as described in the Materials and methods section. The phosphatidylcholine was mixed with the phosphatidylinositol substrate as a chloroform solution and the solvent was evaporated under N_2 . Data are expressed as percentage of duplicate controls with no additions, and are the combined results of four separate experiments.

the effect of phosphatidylcholine on the celery enzyme; we used a celery extract devoid of phospholipase D activity (see above), and checked by using phosphatidyl[^{14}C]choline that no phosphatidylcholine was converted into phosphatidic acid during the assay.

It is apparent from Fig. 2 that phosphatidylcholine will inhibit the celery enzyme, but only partially at a concentration equimolar with phosphatidylinositol. In this respect, therefore, it more closely resembles the lysosomal activity found in mammals (Irvine & Dawson, 1980). Phosphatidic acid, on the other hand, is stimulatory. However, we found over a large number of experiments that the degree of stimulation varied considerably between enzyme preparations. In this response to phosphatidic acid the plant enzyme is more like the cytosolic Ca^{2+} -dependent activity of mammals, though it is less sensitive than the latter enzyme (Irvine *et al.*, 1979b).

The small inhibition by phosphatidylcholine is probably caused by a surface-dilution phenomenon, suggesting that, unlike its mammalian counterpart (Irvine *et al.*, 1979a), the plant enzyme may be able to hydrolyse its substrate in a cell membrane. Preliminary observations with a ^{32}P -labelled microsomal fraction from rat liver as a substrate (Irvine & Dawson, 1978) suggest this is indeed the case.

Other higher plants

We have not embarked on a wide survey of the plant kingdom for the phosphatidylinositol phosphodiesterase found in the present paper in celery; it seems highly unlikely an enzyme probably important in phosphatidylinositol catabolism would be confined to one species only. However, we have examined one other rich source of phospholipase D, the tip of the flower of the cauliflower (*Brassica oleracea*) (Quarles & Dawson, 1969a). In extracts of this tissue phospholipase D is more active against phosphatidylinositol than the phosphodiesterase, and phosphatidic acid is formed in greater amounts at pH 7.0 than diacylglycerol. However, examination of water-soluble products of digestion of [^{32}P]phosphatidylinositol clearly showed release of 1:2-cyclic phosphoinositol and phosphoinositol (though interestingly the latter in slightly larger quantities than the former, a ratio unaffected by a 0.5 mM 1:2-cyclic phosphoinositol 'trap').

We also examined some tissues that have low phospholipase D activities (Quarles & Dawson, 1969a). We could not definitely detect the enzyme in apples (*Pyrus malus*) or potatoes (*Solanum tuberosum*), but by autoradiography of electrophoretic separations of water-soluble products (see the Materials and methods section), we obtained unequivocal evidence (not quantified) for the release of 1:2-cyclic phosphoinositol from [^{32}P]phospha-

tidylinositol by extracts of the outer leaves of onions (*Allium cepa*) and the elongating stem of daffodils (*Narcissus* sp.).

If this enzyme is indeed widespread in the plant kingdom its role in phosphatidylinositol catabolism remains to be established. In mammalian tissues, deacylation seems to be the minor route of phosphatidylinositol breakdown, perhaps to avoid production of the potentially lytic lysophosphatidylinositol (Richards *et al.*, 1979). It is therefore an interesting extension of this possibility that higher plants also have a means of catabolizing phosphatidylinositol without recourse to deacylation.

Celery contains the highest activity of the enzyme of the plants we examined. At a substrate concentration of 50 μM the rate of hydrolysis was of the order of 0.3 nmol/h per mg of tissue (wet weight) at pH 6.6. Direct comparison with the enzyme from rat brain showed the mammalian enzyme to be at least 50 times more active per gram of tissue at its optimum pH (although this enzyme was assayed at 37°C). Such direct comparisons mean little in terms of activities *in vivo*, however (i.e. at physiological pH and temperature); suffice to say that higher plants probably have a much lower phosphatidylinositol phosphodiesterase activity than animals, and this may reflect a role of the plant enzyme in general membrane turnover, compared with the possible additional special function of the animal enzyme in cell activation (Hokin, 1968; Michell, 1975).

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