INOSITOL CYCLIC PHOSPHATE AS A PRODUCT OF PHOSPHATIDYLINOSITOL BREAKDOWN BY PHOSPHOLIPASE C *(BACILLUS CEREUS)*

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1. Introduction

Eukaryotic cells contain inositol phospholipids and also enzymes which cleave the glycerol–phosphate bond in these lipids. The activity of these enzymes within cells is in some way controlled by certain extracellular stimuli, which may thus provoke phosphatidylinositol breakdown. The enzymes which have been studied so far all catalyse breakdown of phosphatidylinositol to 1,2-diacylglycerol and a mixture of inositol 1,2-cyclic phosphate and inositol 1-phosphate, i.e. they act both as cyclizing phosphotransferases and as phosphodiesterases. It has been suggested, but not proved, that inositol cyclic phosphate may be released intracellularly and may function as a second messenger (for a review, see ref. [1]).

The production of inositol cyclic phosphate by the inositide-specific eukaryote 'phospholipase C' might be the result either of a special enzymic mechanism or of the attack of an enzyme with the usual phospholipase C mechanism upon a substrate which can yield a cyclic phosphate. This question cannot be resolved by study of the eukaryote enzymes since they do not attack those lipids which cannot lead to cyclic phosphates. However, the phospholipase C which is present in the culture filtrate of *Bacillus cereus* can be used for such an experiment since it attacks, inter alia, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. The experiments reported here demonstrate that the attack of this enzyme on phosphatidylinositol leads exclusively to production of inositol cyclic phosphate. This enzyme can therefore act either as a phosphodiesterase or as a cyclizing phosphotransferase, the mode of action being determined by the substrate presented to it.

2. Materials and methods

Impure phospholipase C *(Bacillus cereus)* was obtained from Makor Chemicals Ltd., P.O. Box 6570, Jerusalem, Israel (via Digby Chemical Service, 103 Ebury Street, London SW1W 9QV). A highly purified sample, given by Dr R. F. A. Zwaal, was kindly made available by Dr J. B. Finean. Phosphatidylinositol was isolated from liver [2]. Phosphatidylglycerol isolated from Micrococcus lysodeikticus was a gift from Dr V. Long. Myoinositol 1,2-cyclic phosphate and myoinositol 1-phosphate was prepared from phosphatidylinositol by a simple enzymic procedure: this involves treating an ethanol-insoluble fraction of liver phospholipids with a partially purified liver soluble enzyme preparation and separation of the products by chromatography on Dowex 1 × 10 (formate form) using elution by a gradient of ammonium formate (R. H. Michell, in preparation). Glycerol 1-phosphate and glycerol 2-phosphate were from Sigma (London) Chemical Co. Either was converted to glycerol 1,2-cyclic phosphate by treatment with dicyclohexylcarbodiimide in pyridine in a manner analogous to that used for inositol 1,2-cyclic phosphate synthesis [3]. Glycerylphosphorylinositol and glycerylphosphorylglycerol were prepared from their parent lipids by mild alkaline methanolysis [6]. Methods for separation of phosphate esters [4] and of non-polar lipids [5] and other analytical procedures were as used pre-

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viously [2,7]. All of the standard compounds had electrophoretic and chromatographic mobilities similar to those quoted by previous authors [4,6].

3. Results

Phosphatidylinositol was incubated with phospholipase C *(Bacillus cereus)* for periods of 0.5–18 hr, at pH from 5.0–7.0 (10–20 mM Tris–maleate buffers), both in the absence and the presence of Ca^{2+} (up to 2 mM). Phosphatidylinositol breakdown occurred under all conditions but was much more rapid at the lower pH: the reaction rate appeared to be unaffected by Ca^{2+}. Electrophoretic separation of the water-soluble products revealed, under all conditions, only a single phosphorus-containing product. This was eluted from preparative-scale electrophoretic separations, dried, dissolved in water, and examined in detail. It migrated on electrophoresis at pH 3.5 as inositol 1,2-cyclic phosphate and when it was co-electrophoresed with the authentic cyclic phosphate no separation was observed. On treatment with acid (80°C, 3 min, 1 N HCl) it was converted to a phosphorus-containing compound with the mobility of inositol 1-phosphate: this material and authentic inositol 1-phosphate were not separated when they were electrophoresed together. The product from enzymic attack on phosphatidylinositol was not separable from authentic inositol 1,2-cyclic phosphate during chromatography in ethanol–13.5 M ammonia (3:2, v/v) or in propan-2-ol–6 M ammonia (7:3, v/v) [4]. It was clearly separable from glycerylphosphorylinositol, 3-glycerophosphate and inorganic orthophosphate, each of which is a possible (but unlikely) product of phosphatidylinositol breakdown, by one or more of the above procedures.

Examination of the lipid-soluble products of phosphatidylinositol breakdown showed, both at low pH ($\approx$ 5.0) and over long periods of incubation ($\geqslant$ 5 hr), the expected predominance of 1,2-diacylglycerol: much smaller amounts of monoacylglycerol and of unesterified fatty acids were also present. However, if the impure enzyme preparation was used during shorter incubations at or above pH 6.0, 1,2-diacylglycerol did not accumulate and the major products were unesterified fatty acids and some monoacylglycerol. This pH-dependent effect appears to have been the result of lipase activity which was present in the impure enzyme preparations since with the pure enzyme preparation, even at pH 7.0, 1,2-diacylglycerol was the sole lipid-soluble product.

A limited amount of information was also obtained with phosphatidylglycerol; in this case attack was much slower. 1,2-diacyglycerol was again the major lipid-soluble product. The only water-soluble product obtained under any conditions was a phosphomonoester with the electrophoretic mobility of glycerol 1- or 2-phosphate. No evidence for production of glycerol cyclic phosphate was obtained under any conditions of pH or incubation period with either the pure or the impure enzyme preparation. Neither enzyme preparation catalysed breakdown of glycerol cyclic phosphate to glycerol 1- or 2-phosphate under the incubation conditions employed.

Finally, when the pure phospholipase C preparation was presented with an equimolar mixture of phosphatidylglycerol and phosphatidylinositol as substrate it produced a mixture of products which by electrophoresis appeared to consist of glycerol 1- or 2-phosphate and inositol cyclic phosphate.

4. Discussion

The results described above establish, as expected, that phospholipase C preparations from *Bacillus cereus* attack the glycerol–phosphate bond of phosphatidylinositol and phosphatidylglycerol, i.e. that the enzyme preparations attack the same bond in these lipids as in other glycerophospholipids. The observation that phosphatidylinositol and phosphatidylglycerol were attacked by both impure and highly purified phospholipase C preparations suggests that the same enzyme species is probably responsible for attack on these and other glycerophospholipids. The presence of monoacylglycerol and unesterified fatty acids in the lipophilic products released by the impure preparation suggest that this material contained, in addition to phospholipase C, a lipase activity. Like membrane-bound lipases [8,9] this lipase activity could prove troublesome in attempts to interpret the effects of phospholipase C attack upon the properties of membrane preparations.

Although *Bacillus cereus* phospholipase C is, towards most phospholipids, a phosphodiesterase, it attacked phosphatidylinositol by a mechanism which

produced only inositol cyclic phosphate, i.e. the enzyme acted as a cyclizing phosphotransferase. A simple explanation of this observation would be that phosphotransferase activity is preferred but can only occur when the headgroup of the phospholipid substrate included an available hydroxyl group for formation of a phosphodiester bond. However, this is clearly not correct since attack on phosphatidylglycerol did not lead to a cyclic phosphate.

No definitive interpretation of these observations is therefore possible at present, but at least two possibilities seem worth consideration. First, both the hydrocarbon region and the phosphate of all substrates may bind to the enzyme in relatively hydrophobic environments, but with sufficient access of water to the phosphate group for phosphodiesterase activity to occur with almost all substrates: in the case of phosphatidylinositol the large headgroup may block the route of access of water and the enzyme therefore catalyses phosphate bond transfer to the available 2-hydroxyl on the inositol ring. Alternatively, the configurations adopted by the headgroups of phosphatidylinositol and phosphatidylglycerol may differ sufficiently to produce accessibility of a hydroxyl group, and therefore cyclic phosphate formation, only in the case of phosphatidylinositol.

These experiments have shown that production of inositol cyclic phosphate is not a special characteristic of inositide-specific enzymes from eukaryote sources. Phospholipase C from *Bacillus cereus,* which produces only inositol cyclic phosphate, is actually better at generating this product, at least under the conditions we have studied, than the mammalian enzymes which produce $\approx$ 50–90% cyclic phosphate [1,2,7,10]. It may therefore be that the cyclic phosphate is a product of phosphatidylinositol breakdown by all phospholipases C. However, even if this is so it still leaves unexplained the remarkable specificity of eukaryotic phospholipases C towards inositol lipids. This could lie either in the production of inositol cyclic phosphate or in some other effect which depends upon the breakdown of phosphatidylinositol (see ref. [1]). At this stage it should also be remembered that all of the experiments in which inositol cyclic phosphate has been produced by either eukaryote or bacterial enzymes have utilised pure phosphatidylinositol (or detergent-treated membranes [2]) as substrate. These are unnatural situations and investigations of the products of enzymic attack on native membrane preparations is needed.

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