Phosphatidylinositol Cleavage Catalysed by the Soluble Fraction from Lymphocytes

ACTIVITY AT pH5.5 AND pH7.0

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Phosphatidylinositol breakdown by subcellular preparations of small lymphocytes from pig mesenteric lymph nodes was investigated. Activity was higher than in preparations from the tissues studied previously; it was recovered largely in the soluble fraction, which showed pH optima at both 5.4–5.6 and 7.0–7.3. As in other tissues, phosphatidylinositol cleavage produced 1,2-diacylglycerol and a mixture of *myo*-inositol 1:2-cyclic phosphate and *myo*-inositol 1-phosphate. It was stimulated by addition of $CaCl_2$ and, less effectively, by $MgCl_2$. On sucrose-density-gradient ultracentrifugation at pH7.0 two peaks of activity were observed (approx. sedimentation coefficients 8S and 10S); the activity profiles on the gradients were similar when assayed at pH7.0 and 5.5. Activity at pH7.0 (and 0.4mM-$CaCl_2$) was decreased by agents, such as salts and lipophilic cations, which tend to neutralize the negative charge of phosphatidylinositol; at pH5.5 these agents slightly stimulated activity. It is suggested that the same enzyme(s) may be responsible for activity at both pH optima and that previous workers may have underestimated the pH7.0 activity because of the inhibitory influence of cations under the usual assay conditions.

Many types of cell show a rapid enhancement in turnover of phosphatidylinositol during responses to extracellular stimulation. Links have been suggested between this effect and various cellular functions, including secretion (L. E. Hokin, 1968), neurotransmission (Durell *et al.*, 1969; De Robertis, 1271) and long-term adaptive changes (M. R. Hokin, 1968; Kerkof & Tata, 1969; Fisher & Mueller, 1971). Current evidence does not, however, appear to support these suggestions, except possibly for the last (Gerber *et al.*, 1973; Lapetina & Michell, 1973a).

Small lymphocytes are relatively undifferentiated cells which are metabolically rather inactive. Treatment with a variety of agents, including phytohaemagglutinin, induces these cells to change into lymphoblasts which subsequently divide, this process being analogous to the physiological response of sensitized cells to an antigen *in vivo*. Changes in phospholipid metabolism, particularly that of phosphatidylinositol, are among the earliest detected biochemical changes in these stimulated cells (Fisher & Mueller, 1968, 1971; Lucas *et al.*, 1971; Masuzawa *et al.*, 1973). An understanding of these changes might help both to explain some of the subsequent events which occur in stimulated lymphocytes and to indicate a general explanation for enhancement of phosphatidylinositol turnover in stimulated cells.

It seems very probable that control of phosphatidylinositol turnover by extracellular stimuli in a variety of cells is exerted on conversion of phosphatidylinositol into 1,2-diacylglycerol and *myo*-inositol 1:2-cyclic phosphate and/or *myo*-inositol 1-phosphate (Hokin, 1967; Durell *et al.*, 1969; De Robertis, 1971; Michell & Lapetina, 1972; Lapetina & Michell, 1973a,b; Jones & Michell, 1974). Enzyme activities which may be responsible for this reaction occur in soluble fractions from several tissues. (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Friedel *et al.*, 1969; Dawson *et al.*, 1971); in brain there is also considerable membrane-bound activity, which appears to be concentrated in plasma membranes (Canessa de Scarnatti & Rodriguez de Lores Arnaiz, 1972; Michell & Lapetina, 1972; Lapetina & Michell, 1973b). We have therefore examined lymphocytes for the presence of enzymes catalysing the cleavage of phosphatidylinositol. The results have been reported in preliminary form (Allan & Michell, 1973).

Experimental

Materials

Most materials were from sources described previously (Lapetina & Michell, 1973b; Allan *et al.*, 1972). Chlorpromazine hydrochloride was from May and Baker Ltd., Dagenham, Essex, U.K., cinchocaine (also known as dibucaine or nupercaine) from Sandoz Products Ltd., 98 The Centre, Feltham, Middx., U.K., colchicine from Sigma (London)

Chemical Co., Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and vinblastine sulphate from Eli Lilly Ltd., Basingstoke, Hants., U.K.

Methods

Isolation of lymphocytes. The procedure of Allan *et al.* (1972) was modified to allow increased quantities of lymph nodes to be processed. Pig mesenteric lymph nodes were chopped into small pieces (approx. 5mm), mixed with an equal volume of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 11mM-glucose, and gently dispersed by hand by using a loose-fitting (1.5mm clearance) Potter–Elvehjem homogenizer. The resulting dispersion was filtered through muslin and centrifuged at 500*g* for 5min. The sedimented cells were washed three times with 100ml of the above buffer, and the final pellet of cells was resuspended at a concentration of 4×10^8 cells/ml. The final preparation contained mainly small lymphocytes, about 80% of which excluded Trypan Blue, 2.5% erythrocytes and 1–2% non-lymphocytic nucleated cells. About 10^{10} cells were obtained from 50g of tissue.

Subcellular fractionation. Subcellular fractions, except for concentrated soluble fractions, were isolated from lymph nodes by the method of Allan & Crumpton (1970).

Highly concentrated soluble fractions, used as the enzyme source in all of the experiments reported here other than those depicted in Fig. 1, were obtained as follows. Some 6×10^9 cells (approx. 2ml packed volume) were sedimented, washed once with 200mM-KCl and frozen in a solid-CO_2–ethanol mixture. After three cycles of freezing and thawing the treated cells were centrifuged for 1h at $1.5 \times 10^5 g$. The pink supernatant fluid (approx. 1ml) was collected and stored at −20°C. EGTA [ethanedioxybis(ethylamine)-tetra-acetate; 0.5mM final concn.] was added immediately before use of the material. The pellet, when needed, was resuspended in 200mM-KCl by sonication.

Isolation of phosphatidylinositol. Non-radioactive phosphatidylinositol was prepared from pig liver as previously described (Lapetina & Michell, 1973*b*). The ^{32}P-labelled lipid was isolated from ^{32}P-labelled lymphocytes. Carrier-free $^{32}P_i$ (1 mCi) was added to a cell suspension consisting of 1×10^9–2×10^9 lymphocytes in 5ml of phosphate-free Krebs–Ringer bicarbonate buffer. After incubation at 37°C for 1h, 20μg of phytohaemagglutinin (Burroughs-Wellcome, Temple Hill, Dartford, Kent, U.K.) was added in 10μl of 0.9% (w/v) NaCl and incubation was continued for 3h. Lipids were extracted and separated by chromatography on formaldehyde-treated papers (Lapetina & Michell, 1973*b*). The area corresponding to phosphatidylinositol was cut out and eluted with chloroform–methanol (1:2, v/v), the eluate evaporated *in vacuo* and the isolated lipid dissolved in chloro-

form. Approx. 3μCi of [^{32}P]phosphatidylinositol was obtained, with a specific radioactivity of 10Ci/mol. The isolated material moved as phosphatidylinositol on rechromatography and over 95% was susceptible to breakdown by the phosphatidylinositol-specific enzyme preparation used in these experiments. ^{32}P-labelled phosphatidylcholine and phosphatidylethanolamine were isolated from labelled lymphocyte lipids by t.l.c. (Skipski *et al.*, 1964).

Assay of phosphatidylinositol breakdown. The standard assay conditions were as follows. Unlabelled (0.6μmol) and ^{32}P-labelled (approx. 20000d.p.m.) phosphatidylinositol were mixed in a test tube and the chloroform was removed by evaporation. The volume was made up to 0.5ml, the final mixture containing 25mM-Tris–maleate–NaOH buffer, pH7.0 or 5.5 (Gomori, 1955), and 0.4mM-$CaCl_2$. The lipid was dispersed with a vortex mixer and the suspension equilibrated at 37°C on a water bath. Reaction was started by addition of 10μl of the appropriate enzyme preparation and was stopped 5min later by addition of 1.9ml of chloroform–methanol (1:2, v/v). Chloroform (0.6ml) and 2M-KCl (0.6ml) were then added, the tubes mixed and the phases separated by centrifugation. The upper phase was removed and diluted to 10ml with water, and its radioactivity was determined by Cerenkov counting (Lapetina & Michell, 1973*b*). Enzyme concentrations were chosen such that less than 30% of the substrate was destroyed during incubation.

Variation of pH. Tris–maleate–NaOH buffers (25mM in Tris and maleic acid) were used over a pH range of 4.7–8.3 (Gomori, 1955).

Analysis of reaction products. The water-soluble products were identified by high-voltage electrophoresis both before and after acid treatment (Dawson & Clarke, 1972; Lapetina & Michell, 1973*b*). The lipid-soluble products were identified by t.l.c. (Skipski *et al.*, 1968).

Sucrose-gradient centrifugation. Linear gradients of sucrose (5–20%, v/v; total vol. 1.8ml) containing 10mM-Tris–maleate–NaOH buffer, pH7.1, were prepared in 2ml polycarbonate tubes. Samples (100μl) of the lymphocyte soluble fraction, adjusted to pH7.1, were layered on the gradients and the tubes centrifuged at 157000*g* for 4h at 4°C in a 10×10ml angle rotor. Identical gradients, each loaded with a protein of known sedimentation coefficient [rabbit immunoglobulin G ($s_{20,w} = 6.8$S), ovalbumin ($s_{20,w} = 3.6$S) or soya-bean trypsin inhibitor ($s_{20,w} = 2.3$S)], were run simultaneously. Pig haemoglobin contaminating the lymphocyte supernatant provided an additional internal molecular-weight standard ($s_{20,w} = 4.1$S). Gradients were separated into 0.1ml fractions and their enzyme activity (at pH5.5 and 7.1) and protein contents determined. Protein distributions in the

reference gradients were determined spectrophoto-metrically at 280 nm.

Analytical methods. Methods for paper chromato-graphy of lipids and for determinations of phosphate, protein and radioactivity were those used previously (Lapetina & Michell, 1973*b*).

Results

Subcellular distribution of activity

In initial experiments whole lymph nodes were fractionated by the procedure of Allan & Crumpton (1970). Under such conditions approx. 90% of the phosphatidylinositol-cleaving activity was recovered in the soluble fraction (Fig. 1); since the particulate fractions were unwashed the true value may be some-what higher. When the assays were repeated in the presence of 2 mg of deoxycholate/ml, which enhances particulate activity in cerebral-cortex preparations (Lapetina & Michell, 1973*b*), much lower activities were observed in most fractions (Fig. 1). The activity of a plasma-membrane fraction isolated from the 20000*g* pellet was, however, somewhat enhanced by deoxycholate. Subsequent studies were therefore con-cerned with the properties of the activity present in a soluble fraction isolated from cells by a simplified procedure (see under 'Methods').

The activity observed in homogenates of whole lymph nodes was approximately accounted for by the activity measured in an equivalent quantity of iso-
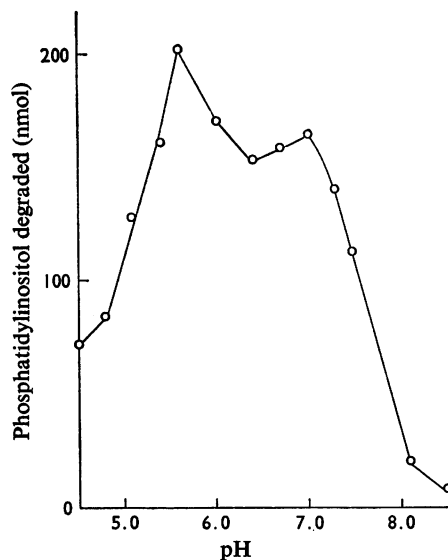

Fig. 2. *Effect of pH on phosphatidylinositol-cleaving activity*

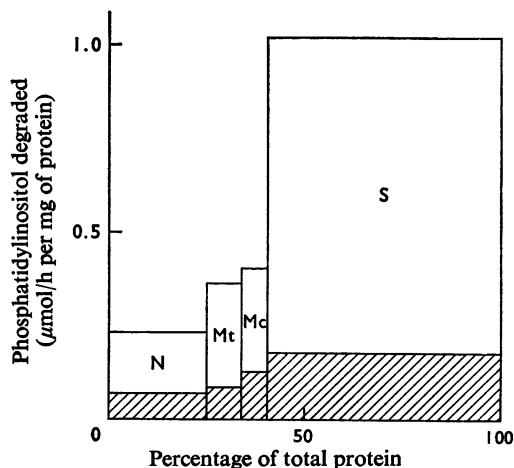Experimental details are given under 'Methods'.

Fig. 1. *Distribution of phosphatidylinositol-cleaving activity at pH 7.0 in subcellular fractions from lymph nodes*

Activity was assayed as described by Lapetina & Michell (1973*b*), both in the presence of 2 mg of sodium deoxy-cholate/ml (shaded area) and in its absence (total area). The fractions are designated N (nuclear), Mt (mitochond-rial), Mc (microsomal), and S (soluble supernatant).

lated cells. Further, erythrocyte lysates, which cer-tainly contributed a significant quantity of protein to the isolated soluble fraction, had no activity.

Effect of pH on activity

Two peaks of activity were observed, one at pH 5.4–5.6 and the other at pH 7.0–7.3 (Fig. 2), the latter usually being slightly lower than the former. Both these activities showed similar behaviour during the isolation procedure: the soluble fraction isolated from lymphocytes was approximately 3-fold enriched with respect to both activities (Table 1).

Time-course

The reason for the difference in time-course at the two pH values, with activity ceasing after about 50% of the phosphatidylinositol had been degraded at pH 7.0 (Fig. 3), is not clear. It should be noted, how-ever, that all assays were done by using 5 min incuba-tions and thus lie in the early part of the curves shown.

Products of phosphatidylinositol breakdown

The major water-soluble product was *myo*-inositol 1:2-cyclic phosphate both at pH 5.4 (55–65% of water-soluble ^{32}P) and at pH 7.3 (50–60% of water-soluble ^{32}P). Its identity was established by high-voltage electrophoresis before and after acid treat-ment (Dawson & Clarke, 1972). The only other major water-soluble product was *myo*-inositol 1-phosphate.

Table 1. *Distribution of phosphatidylinositol-cleaving activity in particulate and soluble fractions from lymphocytes*

For details see the text.

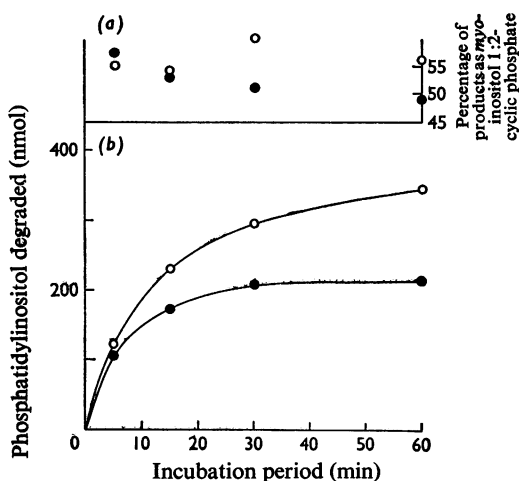|	Protein (mg)	Total activity (μmol/min)		Specific activity (μmol/min per mg of protein)		Relative specific activity	
		pH 5.4	pH 7.3	pH 5.4	pH 7.3	pH 5.4	pH 7.3
Cell pellet (frozen and thawed)	120	10.5	9.8	0.088	0.082	(1.0)	(1.0)
10^8g-min pellet	105	3.5	2.6	0.034	0.025	0.39	0.31
10^8g-min supernatant ('soluble')	23	6.3	5.7	0.28	0.26	3.2	3.2
Recovery	107%	94%	87%				

Fig. 3. *Time-course and products of reaction at pH5.4 (○) and at pH7.4 (●)*

Incubations were stopped at various times, the water-soluble products isolated and their radioactivity was determined by the usual methods, except that water was substituted for 2M-KCl during the extraction procedure. The aqueous solutions containing the reaction products were dried by rotary evaporation, dissolved in 50 μl of water and subjected to high-voltage electrophoresis together with authentic samples of *myo*-inositol 1:2-cyclic phosphate and *myo*-inositol 1-phosphate. The phosphate-containing areas were located, digested in 72% $HClO_4$ and the radioactivity of the resulting solutions was determined (see under 'Methods'). Fig. 3(*a*) shows, at each time and pH, the percentage of the total water-soluble products which was *myo*-inositol 1:2-cyclic phosphate. Fig. 3(*b*) shows degradation of phosphatidylinositol.

The relative proportions of these two compounds did not change appreciably during incubations of up to 1 h (Fig. 3), indicating either that the lymphocyte soluble fraction had little *myo*-inositol 1:2-cyclic phosphate phosphodiesterase activity or that this was inactive under the assay conditions used. P_i was only detected after incubation for 1 h at pH 7.3; even then it

only constituted 2–3% of the total water-soluble products. Glycerylphosphorylinositol was not detected.

The major lipid breakdown product was 1,2-diacylglycerol, with only traces of monoacylglycerol and unesterified fatty acid.

Specificity

Phosphatidylinositol was rapidly attacked, but no activity was detectable towards either phosphatidylcholine or phosphatidylethanolamine.

Density-gradient ultracentrifugation

Two peaks of activity were detected in positions corresponding to sedimentation coefficients of about 8S and 10S. Very similar profiles were obtained with assays carried out at pH 5.5 and 7.0 (Fig. 4). Addition of colchicine (1 mM) or vinblastine (2 mM) to samples before they were applied to the gradient had no effect on their sedimentation behaviour. The products of phosphatidylinositol cleavage at pH 7.0 by the activity in each peak were similar (8S, 55% cyclic phosphate; 10S, 57% cyclic phosphate).

Effects of cations on activity

The usual assay conditions included 0.4 mM-$CaCl_2$, a concentration which gave maximum activation (Fig. 5). Substitution of $MgCl_2$ was only partially effective, especially at low concentrations. The effects of the addition, in the presence of 0.4 mM-$CaCl_2$, of other cations, both hydrophilic (K^+) and amphiphilic (chlorpromazine or cinchocaine), depended on the pH (Fig. 6). At pH 5.5 they were stimulatory, whereas at pH 7.0 they were inhibitory; at the latter pH Ca^{2+} and Mg^{2+} at concentrations above 0.4 mM were also inhibitory.

Stability

The soluble enzyme preparation was stored for several months at $-20°C$ without noticeable loss of activity. Its activity was not decreased by incubation of the undiluted fraction at 37°C for 60 min. The activity was, however, more labile once the concentrated fraction had been diluted.

Effects of phytohaemagglutinin

Soluble fractions prepared from cells treated before homogenization with phytohaemagglutinin (20 μg/ml, pH 7.3, 20 min, 37°C) showed the same activity as equivalent fractions from untreated cells.

Discussion

In most tissues which have been studied previously most of the phosphatidylinositol-cleaving activity was soluble (Kemp *et al.*, 1961; Atherton & Hawthorne,
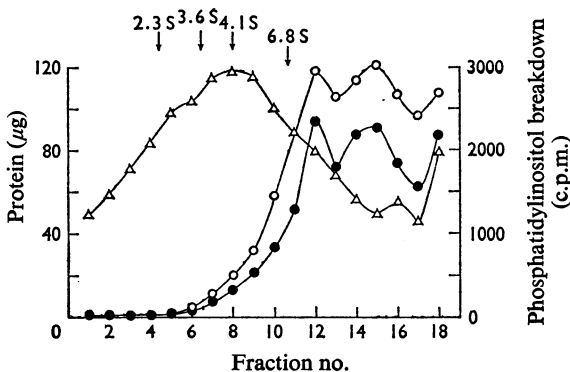

Fig. 4. *Sucrose-density-gradient ultracentrifugation of lymphocyte soluble fraction*

A portion (40 μl) of each fraction was used for assay of enzyme activity at pH 5.5 (○) and at pH 7.1 (●). Protein was determined on 10 μl of each fraction (△). Standards were soya-bean trypsin inhibitor ($s_{20,w}$ = 2.3 S), ovalbumin ($s_{20,w}$ = 3.6 S), pig haemoglobin ($s_{20,w}$ = 4.1 S) and rabbit immunoglobulin G ($s_{20,w}$ = 6.8 S). Values of $s_{20,w}$ for these proteins were obtained from Sober (1970).

1968), although brain appeared to differ by also having considerable membrane-bound activity (Keough & Thompson, 1972; Canessa de Scarnatti & Rodriguez de Lores Arnaiz, 1972; Michell & Lapetina, 1972; Lapetina & Michell, 1973*b*). The lymphocyte seems to follow the former pattern. We have therefore concentrated our studies on the properties of the soluble enzyme(s), using a highly concentrated soluble fraction prepared by a simple one-step ultra-centrifugation of packed lymphocytes. The yield from this method was not quite as high as in the soluble fraction from a conventional cell fractionation, but it was preferred because it gave a fraction of high protein concentration, with salt concentrations rather similar to those within the cell. The successful preparation of this fraction suggests that the 'soluble' nature of the majority of this activity is not likely to be an experimental artifact. Its stability, relative to preparations studied previously (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968), may be because it was maintained under conditions which approximated more closely to those inside a cell.

Several previous workers have commented on the presence in their soluble preparations of appreciable activity at or around pH 7.0 (Kemp *et al.*, 1961; Atherton, 1967; Jungalwala *et al.*, 1971); they have, however, studied primarily the activity at the acid pH optimum. It is clear from the data presented here that in the lymphocyte soluble fraction the activities seen at pH 5.4–5.6 and 7.0–7.3 are quantitatively of similar importance. The assay conditions used in previous studies, which usually combined higher ionic strengths and $CaCl_2$ concentrations than we have used here, were such that activity at pH 7.0 would have been rather depressed; it thus seems likely that under appropriate conditions a high pH 7.0 activity would be detected in other systems. Although
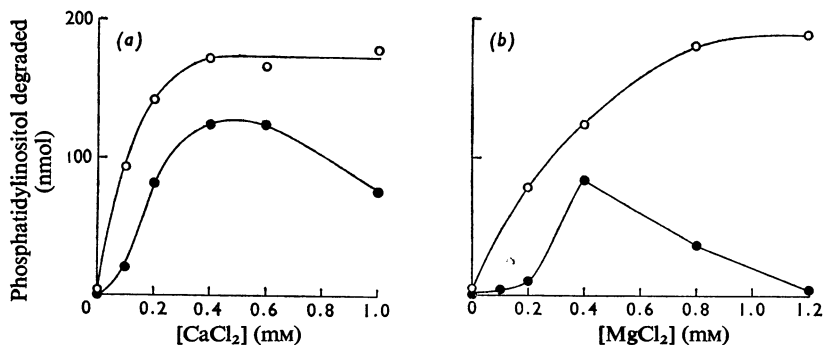

Fig. 5. *Effects of Ca^{2+} and Mg^{2+} concentrations on phosphatidylinositol-cleaving activity*

Different amounts of (*a*) $CaCl_2$ and (*b*) $MgCl_2$ were included in the standard assay medium either at pH 5.6 (○) or at pH 7.1 (●).
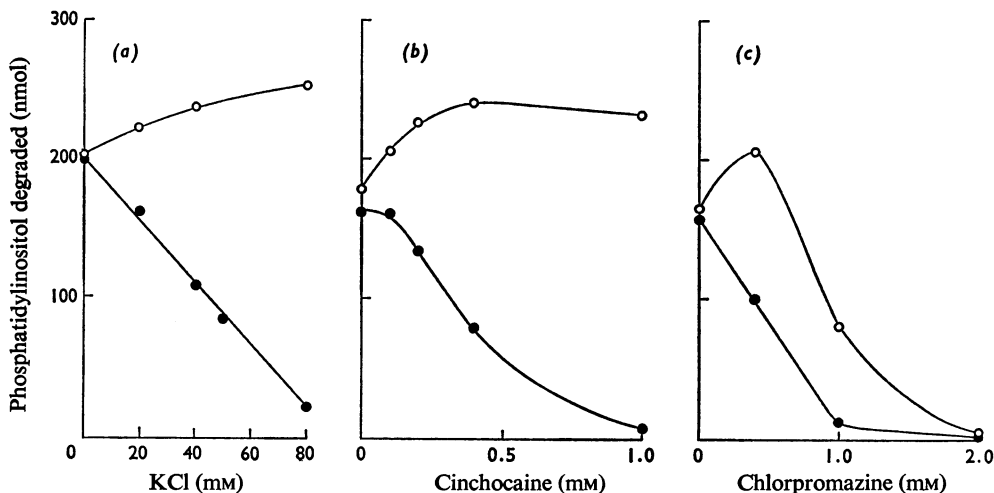
Fig. 6. *Effects of KCl, cinchocaine and chlorpromazine on phosphatidylinositol-cleaving activity*

Different amounts of (a) KCl, (b) cinchocaine hydrochloride, and (c) chlorpromazine hydrochloride were included in the standard assay medium either at pH 5.6 ($\bigcirc$) or at pH 7.1 ($\bullet$).

it is far from certain, it at present seems likely, particularly from the ultracentrifugation experiments, that the activities at pH 7.0 and 5.5 both arise from the action of the same enzyme species. This point is further discussed in the following paper (Allan & Michell, 1974).

Although ionic effects on phosphatidylinositol cleavage have been investigated in previous studies, they have in general led to slightly equivocal conclusions. Usually an indication of a Ca^{2+} requirement has emerged, but there has been some doubt as to the specificity of this effect (Kemp *et al.*, 1961; Atherton & Hawthorne, 1968; Lapetina & Michell, 1973*b*). In consideration of these effects little account has been taken of the high affinity of phosphatidylinositol for Ca^{2+} (Abrahamson *et al.*, 1968; Hauser *et al.*, 1969). Our experiments confirm the stimulatory effect of Ca^{2+}; the specificity of this effect is explored in greater detail in the accompanying paper (Allan & Michell, 1974). Other types of cations (univalent and bivalent hydrophilic, and also amphiphilic) also have effects on the observed activity; they are, in general, moderately stimulatory at pH 5.5 and inhibitory at pH 7.0. The relative affinities of these ions for phosphatidylinositol fall roughly into two groups, with the univalent metal ions about two to three orders of magnitude less tightly bound than Ca^{2+}, Mg^{2+}, chlorpromazine or cinchocaine (Abrahamson *et al.*, 1968; Hauser & Dawson, 1967, 1968). The relative potencies of these ions in modifying the rate of phosphatidylinositol breakdown show a similar pattern. A simple interpretation of this might be that at pH 5.5 the preferred substrate is neutralized phos-

phatidylinositol, whereas at pH 7.0 the negatively charged form of the substrate is attacked more rapidly; the stimulatory effect at pH 5.5 is probably a related effect caused by the release of Ca^{2+} bound to phosphatidylinositol (Allan & Michell, 1974).

It is noteworthy that a quite different enzyme which also uses phosphatidylinositol as substrate, namely the phosphatidylinositol kinase of adrenal chromaffin granules (Phillips, 1973), shows certain similarities in behaviour to the activity described here. In particular, its pH–activity curve shows two maxima, near pH 5.5 and 7.5, and the time-course of its activity is similar, with pH 5.5 activity continuing after the neutral activity has ceased. Possibly both the phosphatidylinositol-cleaving activity and phosphatidylinositol kinase are influenced in similar fashion by the physical state of the substrate. In view of the low pK_a of phosphatidylinositol (approx. 3.5; Abrahamson *et al.*, 1968) it is not clear how pH changes could significantly affect substrate ionization between pH 5.5 and 7.5; the possibility that changes in buffer ionic strength could alter the substrate configuration has not, however, been excluded.

Evidence has been presented by Quinn (1973) on the basis of which he suggested that the soluble enzyme present in the brain (Friedel *et al.*, 1969) may be associated with microtubules. Our limited experiments with colchicine and vinblastine have, however, shown no effects of low concentrations of these agents on the lymphocyte system. It should be emphasized, however, that the molecular weight of our slowest-sedimenting peak of enzyme activity is approximately equivalent to that of the largest form

observed by Quinn (1973); the intestinal-mucosal enzyme is also of about the same size (Atherton & Hawthorne, 1968).

The specific activity of the lymphocyte soluble fraction is severalfold higher than those reported for other systems. Exact comparison is difficult because of the different purifications achieved by other procedures and because we have used isolated cells, rather than a whole tissue, as the starting material. It does, however, seem likely that lymphocytes have a genuinely high intrinsic activity. It is not yet possible to determine whether the enzyme described here is directly involved in the very rapid phosphatidylinositol turnover observed in stimulated lymphocytes (Fisher & Mueller, 1968, 1971). It is, however, notable that sufficient activity is present in these cells to degrade all of their phosphatidylinositol in only a few seconds: the control to which this must be subject both in quiescent cells and in stimulated cells is clearly of considerable importance.

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