Biochem. J. (1983) **212**, 691–697
Printed in Great Britain

The bivalent-cation dependence of phosphatidylinositol synthesis in a cell-free system from lymphocytes

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(*Received 16 February 1983/Accepted 9 March 1983*)

The bivalent-cation requirements of two enzymes involved in phosphatidylinositol synthesis were defined for pig lymphocyte membranes using a citric acid buffer. CTP:phosphatidic acid cytidylyltransferase (EC 2.7.7.41) is activated by free Mn^{2+} concentrations above 20nM and by free Mg^{2+} concentrations above 10μM. When activated by Mg^{2+}, the enzyme is weakly inhibited by Ca^{2+} ($K_i > 250\mu M$), but Ca^{2+} has no effect when Mn^{2+} is used to stimulate CDP-diacylglycerol synthesis. The synthesis of phosphatidylinositol from phosphatidic acid is also stimulated by Mn^{2+} and Mg^{2+} concentrations similar to those above and is inhibited by free Ca^{2+} concentrations above 500nM, probably by its action on CDP-diacylglycerol:inositol 3-phosphatidyltransferase (EC 2.7.8.11). Taken together, these studies suggest that under physiological conditions phosphatidylinositol synthesis is activated by Mg^{2+} and it is possible that it is further regulated by the free concentrations of Ca^{2+} and/or Mn^{2+}.

In many cells and tissues the binding of agonists to plasma-membrane receptors is rapidly followed by an increase in the rate of phosphatidylinositol breakdown (Michell, 1975; Irvine *et al.*, 1982). The mechanism by which this occurs is controversial (Cockcroft, 1981; Hawthorne, 1981; Michell, 1982), but a consequence of phosphatidylinositol breakdown by the Ca^{2+}-activated phosphatidylinositol phosphodiesterase is the production of phosphatidic acid. Subsequent resynthesis of phosphatidylinositol is mediated by the sequential action of CTP:phosphatidic acid cytidylyltransferase (EC 2.7.7.41) and CDP-diacylglycerol:inositol 3-phosphatidyltransferase (EC 2.7.8.11). These membrane-bound enzymes have been extensively studied in cell-free systems derived from several tissues (see, for example, Paulus & Kennedy, 1960; Carter & Kennedy, 1966; Prottey & Hawthorne, 1967) and it is generally accepted that they are stimulated by Mg^{2+} or Mn^{2+}. Carter & Kennedy (1966), Liteplo & Sribney (1980) and Sribney & Hegadorn (1982) reported that CTP:phosphatidic acid cytidylyltransferase of chicken liver, rat liver and pig lymphocytes was maximally activated by Mg^{2+} concentrations in excess of 20mM; similar concentrations of Mn^{2+} supported between 15 and 50% of the Mg^{2+}-stimulated activities. Prottey & Hawthorne (1967), Benjamins & Agranoff (1969), Takenawa & Egawa (1977) and Egawa *et al.* (1981) all found that CDP-diacylglycerol:inositol 3-phosphatidyltransferase in homogenates prepared from several tissues was maximally stimulated by Mn^{2+} concentrations of between 0.5mM and 2mM, whereas 20mM-Mg^{2+} was required for comparable stimulation.

However, in all of the above studies the free concentrations of the bivalent cations (M^{2+}) in the assay media were not buffered and M^{2+}-binding substances (including phosphate, albumin, detergents, CDP-diacylglycerol and phosphatidic acid) were usually added to the tissue homogenates. Consequently, the free M^{2+} concentrations in the assay media were undefined and it is therefore difficult to assess whether these enzymes are likely to be regulated by bivalent cations in the intact cell. In the present study, we have used a citrate buffer for bivalent cations to determine how the free concentrations of Mg^{2+}, Mn^{2+} and Ca^{2+} affect the synthesis of both CDP-diacylglycerol and phosphatidylinositol from endogenous phosphatidic acid in pig lymphocyte membranes.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; M^{2+}, bivalent cation.

Materials and methods

M^{2+}-buffer

Potassium citrate buffer (45mM-tripotassium citrate/31mM-NaCl/7.5mM-Hepes, pH7.1, at 37°C) contains K^+, Na^+, H^+ and Cl^- at concentrations reported for the lymphocyte cytoplasm

(Lichtman *et al.*, 1972; Felber & Brand, 1982; Rink *et al.*, 1982). The free M^{2+} concentrations in the buffer were computed using an iterative program similar to that described by Fabiato & Fabiato (1979). Binding constants for H^+ and M^{2+} for citrate and CTP (or any NTP) were obtained from Martell & Smith (1977). When prepared in Milli-Q purified water (Millipore Corp.) the total M^{2+} concentrations in potassium citrate buffer were typically: Ca^{2+}, 15 μM; Mg^{2+}, 10 μM; Mn^{2+}, <1 μM, as determined by atomic-absorption spectrophotometry. The corresponding free M^{2+} concentrations for Ca^{2+}, Mg^{2+} and Mn^{2+} were calculated as 110 nM, 32 nM and 2 nM. The values for free Ca^{2+} and Mg^{2+} were checked by fluorescence intensity measurements using quin-2 as described in Hesketh *et al.* (1983). The binding of H^+ and each M^{2+} ion to citrate is competitive, and so the addition of any of the cations increases the free concentrations of the other cations and acidifies the buffer. To maintain a constant pH of 7.1, previously determined amounts of Tris base were added with the bivalent cations. In practice, competition between the bivalent cations for citrate was only significant when high total Mg^{2+} concentrations (>20 mM) were used to produce free Mg^{2+} concentrations in the range 0.1–1 mM. CTP was the grade 1 sodium salt of Boehringer, GTP was from Sigma and ATP (purified free of other NTP by the method of Kimura *et al.*, 1976) was a gift from Mrs. E. Kennedy. All other reagents were of AnalaR grade.

Lymphocyte membranes

Mesenteric lymph nodes (175 g) from freshly-slaughtered young pigs were dispersed in 600 ml of medium (116 mM-NaCl, 5.4 mM-KCl, 1 mM-NaH_2PO_4, 11 mM-glucose, 10 mg of Phenol Red/litre, 25 mM-Hepes, pH 7.3) to yield 5×10^{10} cells, which were washed by centrifugation (1000 *g*, 5 min) into an equal volume of 200 mM-KCl. The cells were re-centrifuged and the cell pellets were immediately frozen under liquid N_2 and disrupted in the minimum volume of 200 mM-KCl by three cycles of freeze–thawing followed by three strokes of a Dounce homogenizer at 4°C. The homogenate (50 ml) was fractionated by centrifugation (100000 $g_{av.}$ for 90 min at 4°C); the pale-red supernatant solution and the underlying white layer of membranes were separated and stored on ice. The membrane fraction (packed volume 2 ml) was homogenized in 18 ml of 200 mM-KCl, washed three times by centrifugation (250000 $g_{av.}$ for 15 min at 4°C) and re-homogenized in potassium citrate buffer before dialysis for 15 h at 4°C against three 500 ml portions of the same buffer. The membranes were washed by centrifugation as above, re-homogenized in 9 ml of potassium citrate buffer, frozen in 100 μl portions under liquid N_2 and stored at −25°C. No change in the activity or M^{2+}-sensitivity of either CTP:phosphatidic acid cytidylyltransferase or CDP-diacylglycerol:inositol 3-phosphatidyltransferase occurred during storage for several months. The yields of protein (194 mg) and phospholipid (46 mg) from the membranes (11 ml) were estimated by the methods of Goa (1953) and Rouser *et al.* (1970).

The incorporation of inositol into phosphatidylinositol

Lymphocyte membranes (177 μg of protein, 43 μg of phospholipid) were incubated for 1 h at 37°C in 500 μl of potassium citrate buffer containing the concentrations of CTP, M^{2+} and *myo*-[2-^{3}H]inositol (3.2–16.3 Ci/mmol; Amersham International) described in the Figure legends. The reactions were started by addition of membranes and terminated with 100 μl of 100 mM-EDTA/10 mM-inositol, pH 7.1, and transfer of the tubes to ice. Lipids were immediately extracted by vortex-mixing with 3 ml of chloroform/methanol/12 mM-HCl (100:50:1) and the organic phase was washed four times by vortex-mixing with 2 ml of methanol/1 M-KCl + 10 mM-inositol (1:1) until no radioactivity was detectable in the aqueous phase. The ^{3}H-label in the organic phase, identified as [^{3}H]phosphatidylinositol by high-pressure-liquid-chromatographic analysis (Guerts van Kessel *et al.*, 1977; Moore *et al.*, 1982), was determined by liquid-scintillation counting (efficiency for ^{3}H, 26%). No radioactivity was found in the organic phase if the reactions were terminated immediately after the addition of membranes and no further incorporation of [^{3}H]inositol into [^{3}H]phosphatidylinositol occurred if, after labelling membranes for 1 h, the incubation was continued for a further 15 min at 4°C after addition of 100 mM-EDTA/10 mM-inositol.

The conversion of phosphatidic acid into CDP-diacylglycerol

The procedure was similar to that described for the incorporation of inositol into phosphatidylinositol (membrane concentration 86 μg of phospholipid/500 μl of buffer; [5-^{3}H]CTP sp. radioactivity 19.3 Ci/mmol, from Amersham International; incubations for 15 min at 37°C unless otherwise specified). Reactions were terminated and lipids extracted as described above, except that inositol was omitted from the EDTA solution and from the washes of the organic phase. The ^{3}H-label in the organic phase was identified as [^{3}H]CDP-diacylglycerol by t.l.c. (Hostetler *et al.*, 1976).

Assay of CTP degradation

Membranes were incubated for 15 min or 1 h with various concentrations of [5-^{3}H]CTP, M^{2+} and inositol. After precipitation of the membranes by centrifugation (14000 *g*, 1 minute), 20 μl of the

supernatant solution was analysed by chromatography on polyethyleneimine-impregnated cellulose-coated thin-layer plastic sheets (Camlab, Cambridge, U.K.) with 2M-formic acid/sodium formate (pH 3.4) as solvent (Randerath & Randerath, 1967). Radioactivity in 10mm strips cut from the chromatogram was detected by liquid-scintillation counting and the amounts of [^{3}H]CTP (R_F0.05), [^{3}H]CDP (R_F0.45) and [^{3}H]CMP (R_F0.80) were expressed as a percentage of the total ^{3}H recovered from the chromatogram.

Results

The synthesis of CDP-diacylglycerol

Preliminary experiments confirmed that both Mn^{2+} and Mg^{2+} stimulated the synthesis of CDP-diacylglycerol from CTP and phosphatidic acid in lymphocyte membranes and that the rate of synthesis was unaffected by the addition of 0.1 unit of inorganic pyrophosphatase/ml. When lymphocyte membranes were incubated for 1h with 20μM free Mn^{2+} and a range of [^{3}H]CTP concentrations, the amount of [^{3}H]CDP-diacylglycerol formed was half-maximal at an initial CTP concentration of between 40μM and 50μM. This is comparable with estimates of the CTP concentration in mouse neuroblastoma and human myeloma cells (Khym *et al.*, 1977). However, CTP is degraded to CDP and CMP by phosphatases and/or CTPases present in the membrane preparation and so the K_m for CTP of CTP:phosphatidic acid cytidylyltransferase could not be determined. To avoid significant alteration of the rate of CDP-diacylglycerol synthesis as a result of CTP degradation, a [^{3}H]CTP concentration of 500μM and an incubation time of 15min were chosen as standard conditions. Although the rate of CTP degradation increased with the free M^{2+} concentration, at no concentration of Ca^{2+}, Mn^{2+} or Mg^{2+} used in the experiments described below was more than 30% of the CTP degraded in 15min at this initial CTP concentration. The rate of conversion of CTP (500μM) into CDP-diacylglycerol was constant for 15min and was only 25% lower after 1h. However, at lower CTP concentrations (20μM or 1.2μM) most of the CTP was degraded after a 1h incubation and the rate of CDP-diacylglycerol synthesis was not constant; the amount of [^{3}H]CDP-diacylglycerol labelled was maximal after 75min and 20min respectively, but subsequently decreased, possibly due to the action of hydrolases (Raetz *et al.*, 1972). When [^{3}H]CTP-labelled membranes were washed into fresh buffer containing 20μM free Mn^{2+} (but no CTP) the half-life of [^{3}H]CDP-diacylglycerol was approx. 4h, but when 20μM-inositol was also present, the half-life was reduced to 10min. It is therefore unlikely that CDP-diacylglycerol breakdown catalysed by enzymes other than CDP-diacylglycerol:inositol 3-phosphatidyltransferase is significant in the experiments described below.

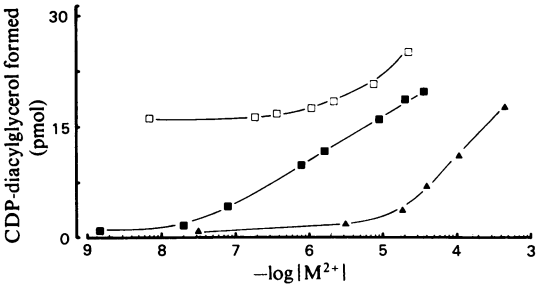

Fig. 1. *The M^{2+}-dependence of CTP:phosphatidic acid cytidylyltransferase*

Lymphocyte membranes were incubated for 15min with 500μM-[5-^{3}H]CTP (40μCi/ml) and the free Mn^{2+} (■) or Mg^{2+} (▲) concentrations indicated or with the free Mn^{2+} concentrations indicated (□) in the presence of approx. 500μM free Mg^{2+}. The free Mg^{2+} concentration increased from 450μM to 580μM over the range of free Mn^{2+} concentrations indicated. N.B. In all of the experiments described in Figs. 1–4, points represent single samples at each M^{2+} concentration. Similar results to those shown in each Figure were obtained in many experiments on several membrane preparations.

The conversion of phosphatidic acid into CDP-diacylglycerol was stimulated by free Mn^{2+} concentrations above 20nM and by free Mg^{2+} concentrations in excess of 10μM (Fig. 1). Stimulation of CDP-diacylglycerol synthesis by free Mn^{2+} concentrations above 1μM was not affected by the presence of 500μM free Mg^{2+}. K_m values from these data were 0.6μM for Mn^{2+} and 40μM for Mg^{2+}; similar K_m values were obtained from experiments in which membranes were incubated for 1h at initial CTP concentrations of 1μM, 20μM and 500μM. All of the CTP was degraded at the lower concentrations, implying that the apparent K_m for M^{2+} of CTP:phosphatidic acid cytidylyltransferase is not affected by the relative concentrations of CTP, CDP and CMP present. Furthermore, the data suggest that the enzyme is activated by free M^{2+} cations, rather than by an M^{2+}–CTP complex.

Ca^{2+} at free concentrations up to 10μM did not stimulate CDP-diacylglycerol synthesis and did not affect synthesis stimulated by 1.7μM free Mn^{2+} at free concentrations up to 40μM. However, CDP-diacylglycerol synthesis stimulated by 470μM free Mg^{2+} was weakly inhibited by Ca^{2+} (Fig. 2). Since 50% inhibition was obtained at a Ca^{2+} concentration above 250μM, Ca^{2+} inhibition of the conversion of phosphatidic acid into CDP-diacylglycerol is unlikely to occur *in vivo*.

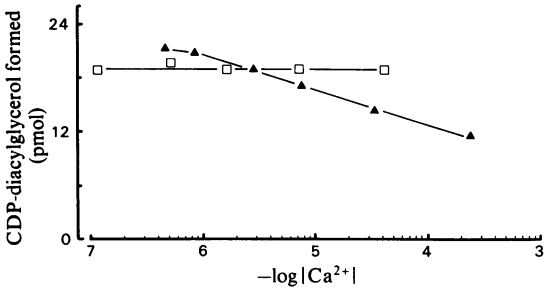

Fig. 2. *The effect of Ca^{2+} on CTP:phosphatidic acid cytidylyltransferase*

Lymphocyte membranes were incubated for 15 min with 500μM-[5-^{3}H]CTP (40μCi/ml) and the free Ca^{2+} concentrations indicated in the presence of 1.7μM free Mn^{2+} (□) or 460μM free Mg^{2+} (▲).

The synthesis of phosphatidylinositol

When lymphocyte membranes were incubated with *myo*-[2-^{3}H]inositol, CTP, and either Mn^{2+} or Mg^{2+}, [^{3}H]phosphatidylinositol was synthesized from endogenous phosphatidic acid in the membranes. The rate of synthesis was linear for 1 h at a CTP concentration of 500μM and an inositol concentration of 6μM, indicating that inositol incorporation into phosphatidylinositol was not affected by the products of CTP degradation.

In most membrane preparations the synthesis of phosphatidylinositol was totally dependent on the addition of CTP. In others, some CTP-independent incorporation of [^{3}H]inositol into [^{3}H]phosphatidylinositol took place at a rate that was up to 20% of that found in the presence of CTP. CTP-independent inositol incorporation, unlike CTP-dependent phosphatidylinositol synthesis, was unstable on storage at −25°C and may be due either to the activity of the Mn^{2+}-dependent inositol-exchange enzyme (Takenawa *et al.*, 1977; Takenawa & Egawa, 1980) or to the condensation of inositol with endogenous CDP-diacylglycerol in the lymphocyte membranes.

The rate of phosphatidylinositol synthesis was half-maximal at initial CTP concentrations of 2.5μM and 10μM in the presence of 20μM free Mn^{2+} and 450μM free Mg^{2+} and maximal at 500μM-CTP in the presence of either ion. Neither ATP nor GTP substituted for CTP, but ATP and GTP concentrations greater than 100μM inhibited phosphatidylinositol synthesis; 50% inhibition was caused by NTP concentrations of 5mM in the presence of 500μM-CTP, 550μM free Mg^{2+} and 7μM free Mn^{2+}. The inhibitory effect of ATP or GTP was not due to reduction of the free M^{2+} concentration (which was buffered), but may be due to competition between the NTP for enzyme binding-sites.

At non-limiting Mn^{2+} (20μM) and CTP (500μM) concentrations the K_m for inositol was 10–30μM in experiments on different membrane preparations. This is considerably less than estimates (1–10mM) of the inositol concentration in several types of mammalian cells (Dawson & Freinkel, 1961; Palmano *et al.*, 1977). The intracellular inositol concentration is therefore unlikely to be limiting for phosphatidylinositol synthesis *in vivo*. The $V_{max.}$ for phosphatidylinositol synthesis was 11pmol/μg of phospholipid per h, whereas the maximum amount of phosphatidylinositol that could be synthesized on prolonged incubation of the membranes with inositol, CTP and Mn^{2+} was 15pmol/μg of phospholipid. The latter value (equivalent to 1.1mol% of the total membrane phospholipid) provides an estimate of the phosphatidic acid content of the lymphocyte membrane preparation.

The rate of conversion of phosphatidic acid into phosphatidylinositol was considerably greater than the rate of synthesis of CDP-diacylglycerol. Under comparable conditions (500μM-CTP, 20μM free Mn^{2+}, 15minute incubation), the rate of CDP-diacylglycerol synthesis was 1.0pmol/μg of phospholipid per h, whereas the rate of phosphatidylinositol synthesis was 2.7 and 10.5pmol/μg of phospholipid per hour at inositol concentrations of 6μM and 100μM respectively. This implies that the rate-limiting step in phosphatidylinositol synthesis is the formation of CDP-diacylglycerol. Once formed, this lipid is rapidly removed by the action of CDP-diacylglyerol:inositol 3-phosphatidyltransferase. A similar conclusion was reached by Prottey & Hawthorne (1967) from experiments using homogenates of guinea-pig pancreas. The steady-state level of CDP-diacylglycerol in tissues is low [approx. 1% of the level of phosphatidic acid (Thompson & MacDonald, 1975, 1976)]. There are several possible explanations for the low rate of CDP-diacylglycerol synthesis in the absence of inositol. For example, inositol may be an activator of CTP:phosphatidic acid cytidylyltransferase, or CDP-diacylglycerol may inhibit its own synthesis via a feedback mechanism.

The effect of M^{2+} concentrations on phosphatidylinositol synthesis

Phosphatidylinositol synthesis was stimulated by Mn^{2+} concentrations above 10nM and by Mg^{2+} concentrations above 10μM (Fig. 3). Variation of the inositol concentration in the range 0.2–20μM, and the CTP concentration in the range 0.5–5mM, did not affect the K_m for M^{2+} of phosphatidylinositol synthesis, but the $V_{max.}$ increased with the inositol concentration. The K_m for Mn^{2+} varied between 0.3μM and 0.8μM and the K_m for Mg^{2+} between 70μM and 165μM, in experiments on different membrane preparations. Although Mn^{2+} was considerably more effective than Mg^{2+} at stimulating

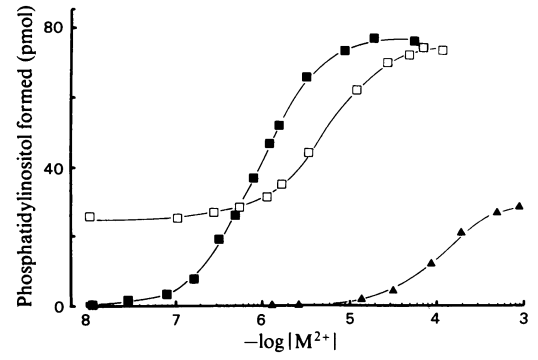

Fig. 3. *The M^{2+} requirement for phosphatidylinositol synthesis*

Lymphocyte membranes were incubated for 1 h with 500μM-CTP, 5.8μM-*myo*-[2-^{3}H]inositol (5μCi/ml) and the free Mn^{2+} (■) or Mg^{2+} (▲) concentrations indicated or with the free Mn^{2+} concentrations indicated (□) in the presence of approx. 1 mM free Mg^{2+}. The free Mg^{2+} concentration rose from 0.8 mM to 1.8 mM over the range of free Mn^{2+} concentrations indicated.

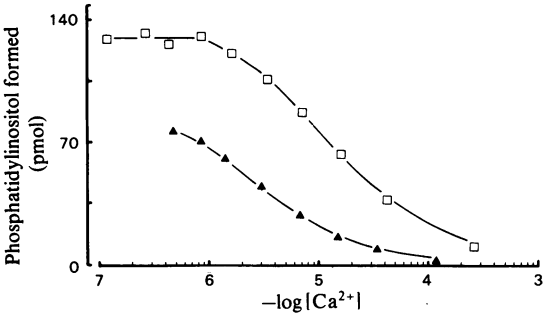

Fig. 4. *The effect of Ca^{2+} on phosphatidylinositol synthesis*

Lymphocyte membranes were incubated for 1 h with 500μM-CTP, 6.1μM-*myo*-[2-^{3}H]inositol (5μCi/ml) and the free Ca^{2+} concentrations indicated in the presence of 1.7μM free Mn^{2+} (□) or 460μM free Mg^{2+} (▲).

phosphatidylinositol synthesis, inositol incorporation into phosphatidylinositol was activated by free Mg^{2+} concentrations of 0.25–1 mM, which are probably typical of the mammalian cell (see the Discussion section). Phosphatidylinositol synthesis stimulated by 1 mM free Mg^{2+} was further potentiated by Mn^{2+}: the K_m for Mn^{2+} was increased to 5.5μM but the $V_{max.}$ of the reaction was unaltered, suggesting that Mg^{2+} is a competitive inhibitor of Mn^{2+}-stimulated phosphatidylinositol synthesis.

Ca^{2+} inhibited phosphatidylinositol synthesis stimulated by Mn^{2+} or by Mg^{2+} (Fig. 4). Synthesis was inhibited by 50% by concentrations of added Ca^{2+} from 2μM to 20μM, irrespective of the activating concentrations of Mn^{2+} or Mg^{2+}. The inhibitory effect of Ca^{2+} on Mn^{2+}-stimulated synthesis was similar whether the CTP concentration was 500μM or 5 mM. In contrast, at 5 mM-CTP there was very little inhibition of Mg^{2+}-stimulated synthesis by Ca^{2+}. Ca^{2+} is a mixed inhibitor of Mn^{2+}-stimulated phosphatidylinositol synthesis; as the free Ca^{2+} concentration was increased from 0.1μM to 15μM, the K_m for Mn^{2+} increased from 0.8μM to 2.2μM, whereas the $V_{max.}$ decreased from 1.8 to 1.6 pmol/μg of phospholipid per h. Since the synthesis of CDP-diacylglycerol was not inhibited by Ca^{2+} (Fig. 2), either Ca^{2+} inhibits CTP:phosphatidic acid cytidylyltransferase only under conditions in which CDP-diacylglycerol:inositol 3-phosphatidyltransferase is also operating, or else the Ca^{2+}-sensitive enzyme is CDP-diacylglycerol:inositol 3-phosphatidyltransferase. Egawa *et al.* (1981) reported that Ca^{2+} inhibited the latter enzyme, activated by Mn^{2+} or Mg^{2+}, in homogenates of aorta and vas deferens and Daniels & Palmer (1980) obtained similar data from studies on microsomes from *Crithida Fasciculata*. Prottey & Hawthorne (1967) found that Ca^{2+} inhibited the Mn^{2+}-stimulated conversion of phosphatidic acid to phosphatidylinositol in guinea-pig pancreas homogenates, in the presence or absence of Mg^{2+}.

At a constant free Mn^{2+} concentration (2μM), the rate of CTP-dependent phosphatidylinositol synthesis increased with the pH of the incubation; the rate of synthesis was 4-fold greater at pH 7.6 than at pH 6.6.

Discussion

The free Mg^{2+} concentration in the cytoplasm of lymphocytes and other mammalian cells is reported to be within the range 0.25–1 mM (e.g., Gupta & Yushok, 1980; Rink *et al.*, 1982) and there is no evidence to suggest that the free Mg^{2+} concentration is substantially lower in any intracellular organelle. It is therefore very likely that the enzymes of phosphatidylinositol synthesis are activated by Mg^{2+} irrespective of their location or orientation with respect to the cytoplasm, since they are found to be activated by free Mg^{2+} concentrations above 20μM.

It is less clear whether, or to what extent, the synthesis of phosphatidylinositol will be modulated by Ca^{2+} and Mn^{2+}, which have opposing effects on the rate of synthesis. Modulation of phosphatidylinositol synthesis by Ca^{2+} and Mn^{2+} in the intact cell will depend critically on their relative con-

centrations at the site of phosphatidylinositol synthesis. Although it has been proposed that phosphatidylinositol is synthesized in the endoplasmic reticulum (e.g., Michell, 1975), it is uncertain whether the enzymes of phosphatidylinositol synthesis are exposed to the cytosol [as for phosphatidylcholine and phosphatidylethanolamine synthesis (Butler & Morell, 1982)] or to the internal ionic environment of the endoplasmic reticulum.

The Ca^{2+} concentration inside the endoplasmic reticulum or other organelles is not known in intact cells, but is likely to be substantially higher than the concentration in the cytosol. The free Ca^{2+} concentration in the lymphocyte cytosol has been estimated as approx. 100nM (Hesketh *et al.*, 1983) and an increase in the intracellular free Ca^{2+} concentration would inhibit phosphatidylinositol synthesis from phosphatidic acid. Furthermore, one of the enzymes of phosphatidylinositol breakdown, phosphatidylinositol phosphodiesterase, is activated by Ca^{2+} concentrations in the range 0.1–1 μM (Allan & Michell, 1974; Hirasawa *et al.*, 1982). We conclude from the studies *in vitro* that an increase in the free cytoplasmic Ca^{2+} concentration in this range will cause a decrease in the phosphatidylinositol content of the cell. It should be noted, however, that this is not incompatible in itself with the frequently-observed increases in incorporation of $[^{32}P]P_i$ or radiolabelled inositol into the phosphatidylinositol pool, which are associated with elevation of the cytoplasmic Ca^{2+} concentration in intact cells.

The free Mn^{2+} concentration has been estimated by Williams (1982) as "likely to be around 10^{-5}M in many vesicular spaces and considerably less than this in the cytoplasm". The free Mn^{2+} concentration in normal rat hepatocytes was reported by Ash & Schramm (1982) as 0.71 μmol/litre of cell water in a total Mn^{2+} concentration of 34.4 μmol/litre of cell water. However, the measurements do not allow free Mn^{2+} in the cytosol and in other cell compartments to be distinguished. Although there has been speculation concerning the role of Mn^{2+} as a metabolic regulator (Williams, 1982; Schramm, 1982), we know of only one other demonstration of the activation of an enzyme [phosphoenolpyruvate carboxykinase (Schramm *et al.*, 1981)] by concentrations of free Mn^{2+} that may be in the physiological range.

Thus it would appear at present that both Ca^{2+} and Mn^{2+} may be physiological regulators of phosphatidylinositol synthesis whether the synthetic enzymes are oriented towards the cytosol or an intravesicular space, but this will remain an open question until the orientation of the enzymes and the relevant free concentrations of Ca^{2+} and Mn^{2+} are determined. We note, however, that modulation of phosphatidylinositol synthesis may depend on the relative Ca^{2+}, Mn^{2+} and Mg^{2+} concentrations rather than their absolute values.

This work was supported by grants from the S.E.R.C. to J.C.M. J.P.M. was the recipient of an M.R.C. Research Studentship. We thank Dr. A. N. Corps for the computer program used to calculate free M^{2+} concentrations and Mr. R. W. Plumb for technical assistance.

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