'Phosphatido-peptide'-like Complexes Formed by the Interaction of Calcium Triphosphoinositide with Protein

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1. The sodium salt of triphosphoinositide partitions in the upper polar phase in a biphasic chloroform-methanol-water system similar to that of Folch *et al.* (1957).
2. On the addition of $2\mu\text{g. atoms}$ of Ca^{2+} or Mg^{2+} per μmole of triphosphoinositide the phospholipid passes entirely into the lower non-polar phase as the dicalcium or dimagnesium salt.
3. When serum albumin is included in the biphasic system, some of the dicalcium (dimagnesium) triphosphoinositide becomes attached to the protein material at the interface.
4. The affinity of Ca^{2+} for triphosphoinositide is 2-2.5 times as great as that of Mg^{2+} and the salt is not dissociated appreciably by equimolar amounts of EDTA or cyclohexane-1,2-diaminetetra-acetate.
5. When dicalcium triphosphoinositide is mixed with serum albumin a complex is formed which is insoluble in chloroform-methanol (2:1, v/v) but which dissolves completely when 0.25% (v/v) of concentrated hydrochloric acid is added.
6. On homogenizing a chloroform-methanol solution of dicalcium triphosphoinositide with guinea-pig liver the phospholipid becomes quantitatively attached to the insoluble residue, but it can be completely extracted from this with acidified chloroform-methanol.
7. The relevance of these observations to the significance of the phosphatido-peptide-complexes extracted from brain is discussed.

The term phosphatido-peptide was coined to describe certain phospholipid complexes in brain tissue which unlike the bulk of the other phospholipids were not extractable by chloroform-methanol mixtures but were soluble in such mixtures acidified with hydrochloric acid (Folch, 1952; LeBaron & Folch, 1956; LeBaron & Rothleder, 1960). The phospholipid concerned was shown to contain inositol and it was originally thought to be tightly bound to a peptide or, more recently, a protein (LeBaron & Rothleder, 1960). Dittmer & Dawson (1961) identified the phospholipid as a triphosphoinositide and this was confirmed by LeBaron (1963).

The original 'diphosphoinositide' isolated by Folch (1949*a,b*) and now known to consist predominantly of triphosphoinositide (Dawson & Dittmer, 1961; Bockerhoff & Ballou, 1961) contained appreciable amounts of magnesium and calcium, with little sodium or potassium. Recently the polyphosphoinositide fraction of brain isolated by countercurrent distribution was found to contain these alkaline-earth metals at a concentration which was roughly equivalent to the monophosphate ester groups present in the phospholipid (Kerr, Kfoury & Djibelian, 1963). It has been suggested that this affinity for Ca^{2+} and Mg^{2+} may play a role in the active transport of these cations in tissues (Hawthorne, 1964).

Triphosphoinositide has recently been identified as a component of brain myelin, and in such a state the phospholipid is completely soluble in chloroform-methanol (Eichberg & Dawson, 1965). This different solubility behaviour of triphosphoinositide in myelin compared with whole brain suggested an investigation into the question whether phosphatido-peptide-like complexes might be formed from triphosphoinositide alkaline-earth metals and proteins under the conditions usually used for their preparation. In addition the affinity of triphosphoinositide for the alkaline-earth metals has been investigated in some detail with a biphasic non-polar solvent system similar to that of Folch, Lees & Sloane-Stanley (1957).

EXPERIMENTAL

Triphosphoinositide. Triphosphoinositide (sodium salt) was isolated from ox brain by the procedure of Dittmer & Dawson (1961) and stored in chloroform solution at -18° .

Determinations. Triphosphoinositide was assayed by the acid hydrolysis procedure of Dawson & Eichberg (1965). Phosphorus was estimated by the method of Fiske & Subbarow (1925). The concentrations of calcium and magnesium in solutions were checked by EDTA titrations (Hildebrand & Reilly, 1957). ^{45}Ca radioactivity was measured by taking a portion of the solution to dryness and oxidizing the organic matter in the residue with 1 ml. of

perchloric acid (Dawson, 1960). The digest was diluted to 10ml. and assayed in a M8 liquid counter tube. The efficiency of this procedure was poor, but since there was no shortage of counts in the system the convenience of the method outweighed this disadvantage.

RESULTS

Distribution of triphosphoinositide and metal ions in biphasic systems formed from chloroform-methanol and an aqueous solution. When a chloroform-methanol (2:1, v/v) solution of sodium triphosphoinositide ($0.7\mu\text{mole}$ in 4ml.) was shaken with 1ml. of water, forming a biphasic solvent system similar to that of Folch *et al.* (1957), the lipid was found to be almost entirely in the upper aqueous methanol phase. When the chloroform-methanol solution was shaken instead with solutions of 2mM-magnesium chloride, 2mM-calcium chloride or protamine sulphate (2.5mg./ml.) the phospholipid was found exclusively in the lower chloroform-rich phase. The addition of sodium chloride or potassium chloride also caused a redistribution of triphosphoinositide into the lower phase, but these salts were very much less effective than the alkaline-earth metal chlorides and even at 0.35M some 20% of the phospholipid remained in the upper aqueous phase.

Fig. 1. Effect of adding CaCl_2 on the distribution of triphosphoinositide in a biphasic solvent system (chloroform-methanol-water, 8:4:3, by vol.). A solution of triphosphoinositide in chloroform-methanol (2:1, v/v; 4ml.) was shaken with 1ml. of aq. CaCl_2 solution (a) and (lower curves; b) with 5mg. of serum albumin added to the system. O, P in lower phase; ●, P in upper phase; Δ, P in interface.

The effect of calcium concentration on the distribution of triphosphoinositide between the two phases is shown in Fig. 1(a). The addition of calcium chloride caused a progressive and roughly proportional movement of the phospholipid to the lower phase, which was essentially complete when 2 atoms of calcium had been added for every molecule of triphosphoinositide ($0.59\mu\text{mole}$ of triphosphoinositide, $1.26\mu\text{g. atoms}$ of calcium). Very similar results were found when magnesium chloride was added to the aqueous phase. With much higher concentrations of calcium chloride (up to 0.4M in the aqueous phase added) the triphosphoinositide still remained in the chloroform-rich phase.

Measurements were made of the distribution of radioactive calcium in the same system on progressively increasing the calcium concentration. The results show that when sufficient calcium had been added to cause all of the triphosphoinositide to pass into the lower phase ($1.5\mu\text{g. atoms}$ of calcium, $0.73\mu\text{mole}$ of triphosphoinositide) all of the calcium was in the lower phase (Fig. 2). At lower concentrations of Ca^{2+} , a small but significant proportion of the bivalent cation remained in the upper polar phase. At higher added concentrations of calcium, no more of the cation passed into the lower phase and it accumulated in the aqueous methanol upper phase (Fig. 2).

The effect of adding magnesium to the distribution of $^{45}\text{Ca}^{2+}$ between upper and lower phases is shown in Fig. 3. Just sufficient $^{45}\text{Ca}^{2+}$ was added to the system to ensure that in the absence of magnesium all of the triphosphoinositide and calcium would be present in the lower phase. The progressive addition of magnesium to the system caused some loss of radioactivity to the upper phase

Fig. 2. Effect of calcium concentration on the distribution of triphosphoinositide and calcium in a biphasic solvent system (chloroform-methanol-water, 8:4:3, by vol.). A solution of triphosphoinositide ($0.73\mu\text{mole}$) in chloroform-methanol (2:1, v/v; 4ml.) was shaken with 1ml. of aq. $^{45}\text{CaCl}_2$ solution. O, P in lower phase; Δ, Ca in lower phase; ●, P in upper phase; □, Ca in upper phase.

Fig. 3. Percentage of calcium present in a triphosphoinositide-alkaline-earth metal salt formed from solutions containing Mg^{2+} and Ca^{2+} . A solution of triphosphoinositide ($0.7 \mu\text{mole}$) in 4 ml. of chloroform-methanol (2:1, v/v) was shaken with 1 ml. of a solution containing $1.5 \mu\text{mole}$ of $^{45}\text{CaCl}_2$ and various amounts of Mg^{2+} . Radioactivity in the lower phase was used as a measure of the Ca^{2+} in the bivalent alkaline-earth metal salt. $\circ$, Actual percentage; $\bullet$, predicted percentage if affinities of Ca^{2+} and Mg^{2+} for triphosphoinositide were equal.

but considerably less than that predicted if it were assumed that Ca^{2+} and Mg^{2+} had an equal affinity for the phospholipid. This situation was not changed appreciably by using buffers of various pH values (5 mM-sodium acetate-acetic acid, pH 5.8, tris-hydrochloric acid, pH 7.2 and pH 9).

Chelators of Ca^{2+} were added to a system which contained just sufficient of the metal to ensure that the triphosphoinositide was present in the lower chloroform-rich phase. At pH 7.2, ethylenediamine-tetra-acetic acid at a molar concentration some seven times as great as that of the triphosphoinositide only caused about a 30% dissociation of the dicalcium triphosphoinositide complex as judged by the phospholipid appearing in the upper aqueous phase (Fig. 4). The more powerful chelating agent cyclohexane-1,2-diaminetetra-acetic acid was more effective at destroying the complex and at a molar concentration five to six times as great as the triphosphoinositide present in the system all the phospholipids had reverted to the upper aqueous phase (Fig. 4).

Formation of complexes between triphosphoinositide, calcium and protein. When crystalline serum albumin (5 mg./ml.) was included in the aqueous solution added to the chloroform-methanol (2:1, v/v) the

Fig. 4. Effect of calcium-chelating agents on the calcium salt of triphosphoinositide. A solution of triphosphoinositide ($0.65 \mu\text{mole}$) in 4 ml. of chloroform-methanol (2:1, v/v) was shaken with 1 ml. of mM-tris-HCl buffer solution (pH 7.2) containing $1.2 \mu\text{moles}$ of $CaCl_2$ and various amounts of chelating agents. $\circ$ — $\circ$, Lower phase, EDTA; $\bullet$ — $\bullet$, upper phase, EDTA; $\circ$ — $\circ$, lower phase, cyclohexane-1,2-diaminetetra-acetate; $\bullet$ — $\bullet$, upper phase, cyclohexane-1,2-diaminetetra-acetate.

progressive addition of Ca^{2+} caused a loss of triphosphoinositide from the upper phase similar to that occurring in the absence of the protein (Fig. 1b). However, the interface between the two phases was now marked by a layer of proteinaceous material which on analysis was found to contain an appreciable amount of the triphosphoinositide (Fig. 1b). Thus the dicalcium triphosphoinositide in the chloroform-rich phase had an appreciable affinity for the protein at the interface. Moreover, the interface formed a compact white skin which was distinctly different from the loose fluffy interface material formed from serum albumin and calcium chloride in the absence of triphosphoinositide.

Attempts were therefore made to form a complex between calcium triphosphoinositide and serum albumin with solubility properties equivalent to the phosphatido-peptides of tissues. Sodium triphosphoinositide ($0.65 \mu\text{mole}$) was dissolved in 1 ml. of water together with 5 mg. of serum albumin and calcium chloride ($1 \mu\text{mole}$). On the addition of 20 vol. of chloroform-methanol (2:1, v/v), a compact easily centrifuged precipitate was obtained containing all of the phospholipid and Ca^{2+} . No lipid phosphorus was removed from the precipitate on further extraction with chloroform-methanol (2:1, v/v). No precipitate was formed in the absence of serum albumin and in the absence of Ca^{2+} little phospholipid was present in the light fluffy precipitate obtained. On the addition of chloroform-methanol (2:1, v/v) containing 0.25% (v/v) of conc. hydrochloric acid the complex immediately dissolved with the formation of a clear solution. A

complex showing essentially the same solubility behaviour was formed when cytochrome *c* was used instead of serum albumin but not with protamine sulphate.

Experiments were made to test whether a tissue homogenate could provide the protein necessary for forming a calcium triphosphoinositide-protein complex. To do this a tissue was chosen that contains virtually no natural triphosphoinositide (Dawson & Eichberg, 1965). Guinea-pig liver (1g.) was homogenized in a blender with 21 ml. of chloroform-methanol (2:1, v/v) containing 0.33 μ mole of triphosphoinositide and 0.5 μ mole of calcium chloride. The residue obtained on centrifuging was extracted with a further 10 ml. of chloroform-methanol (2:1, v/v). The combined extracts contained the bulk of the tissue phospholipids but no triphosphoinositide. The residue was then extracted twice with 10 ml. of chloroform-methanol (2:1, v/v) containing 0.25% (v/v) of conc. hydrochloric acid. The added triphosphoinositide was recovered quantitatively from these extracts. No triphosphoinositide was found in parallel experiments in which none of this phospholipid has been added to the system. In the absence of the liver tissue the triphosphoinositide-calcium salt was completely soluble in chloroform-methanol (2:1, v/v). Liver tissue therefore provided a substance (protein) which combined with the calcium triphosphoinositide forming a complex which was insoluble in neutral chloroform-methanol solvent but completely soluble in acidified chloroform-methanol. When a similar complex was formed and extracted by the multiple extraction procedure of LeBaron & Rothleder (1960) some triphosphoinositide was recovered in the phosphatido-peptide fraction but this was by no means quantitative (33%).

DISCUSSION

The sodium salt of triphosphoinositide dissolves in water giving a clear solution and electron microscopy suggests that the micromicelles present in such solutions consist of only a few molecular units (Thompson & Dawson, 1964). Presumably the presence of five negative charges and two free hydroxyl groups in each molecule confers sufficient polarity to overcome any tendency for the fatty acid hydrocarbon chains of adjacent molecules to come together and form a water-insoluble phospholipid. In the presence of high concentrations of univalent cations (Na^+ , K^+) or lower concentrations of bivalent cations (Mg^{2+} , Ca^{2+}) or protamine the negative charges on the phospholipid would become masked by counter ion-binding and salt formation resulting in the phospholipid becoming more hydrophobic in character. This would account for its precipitation from aqueous solution by Ca^{2+} or

Mg^{2+} and protamine (Thompson & Dawson, 1964) and its redistribution into the non-polar phase of a biphasic solvent system observed on adding Ca^{2+} and Mg^{2+} in the present work and in the counter-current isolation procedure of Kerr *et al.* (1963).

The calcium or magnesium required to cause all of the triphosphoinositide to partition in the non-polar phase of a biphasic system similar to that of Folch *et al.* (1957) is quite specific and amounts to 2 equiv. of magnesium or calcium per molecule of triphosphoinositide. When this amount of ^{45}Ca is added to the system the radioactivity is recovered quantitatively in the lower non-polar phase. Presumably the triphosphoinositide partitions in the lower phase as the dicalcium salt with its monophosphoester groups electrically neutral. The observation that at lower ^{45}Ca /triphosphoinositide concentration ratios a little calcium remains in the upper phase could be accounted for by assuming that a little of the monocalcium salt partitions in the polar phase.

Clearly the affinity for alkaline-earth metals is very high. At pH 7.2 triphosphoinositide can compete effectively with EDTA for Ca^{2+} and Mg^{2+} and even the more effective chelating agent cyclohexane-1,2-diaminetetra-acetate is required at five times the concentration of triphosphoinositide to dissociate the salt completely. This high affinity for Mg^{2+} and Ca^{2+} explains the dramatic effect of these cations on the ion-exchange chromatography of polyphosphoinositides (Hendrickson & Ballou, 1964). The experiments measuring the calcium content of the triphosphoinositide salt in the presence of calcium-magnesium mixtures show that the phospholipid's affinity for calcium is some 2–2½ times as great as that towards magnesium. Possibly this is due to a more thermodynamically stable stereochemical configuration for the dicalcium complex. In this connexion, ionic radii of calcium and magnesium are substantially different (Pauling, 1940) although less so in the hydrated state (Stokes & Robinson, 1948). The degree of hydration depends, of course, to some extent on the nature of the anion and with triphosphoinositide is likely to be low. Calcium is also more readily adsorbed than magnesium on carboxylate surfaces (Goddard & Achilli, 1963), due to an energy of specific adsorption which is higher for Ca^{2+} than Mg^{2+} (Davies & Rideal, 1961; Davies, 1964).

The results clearly show that there is considerable affinity between the dicalcium and dimagnesium salts of triphosphoinositide and protein. In the biphasic solvent system some of the phospholipid salt comes out of the solution in the lower non-polar phase and attaches itself to the denatured protein material at the interface. Moreover, when dicalcium triphosphoinositide salt is mixed with a protein such as serum albumin it forms a complex

which is insoluble in chloroform-methanol but which dissolves readily in chloroform-methanol acidified with hydrochloric acid. These are exactly the solubility properties described for the phosphatido-peptide complexes isolated from brain tissue (Folch, 1952; LeBaron & Folch, 1956) and which are now known to contain triphosphoinositide. The question therefore arises whether phosphatido-peptides as isolated exist in tissues as moieties with physiological meaning or whether they are formed artifactually during the homogenization of the tissue with fat solvents such as chloroform-methanol. The results presented can give no absolute answer although they definitely show that complexes with the same solubility properties can readily be formed from dicalcium triphosphoinositide and certain proteins which are definitely not normally part of a phosphatido-peptide complex. Moreover, such complexes are readily formed by homogenizing a chloroform-methanol solution of calcium triphosphoinositide with guinea-pig liver, a tissue which does not contain any of this phospholipid.

LeBaron (1963) envisages a binding between the phosphatido-peptide complex and the insoluble proteins of the tissue residue which confers insolubility in chloroform-methanol. This linkage is broken by adding acid to the solvent and the phosphatido-peptide dissolves. The present results would suggest that this solubility change could be brought about by the acid-removing alkaline-earth metals from a phosphatido-protein complex. The complete solubility of myelin triphosphoinositide in chloroform-methanol (Eichberg & Dawson, 1965) also argues against the linkage between phosphatido-peptide and protein existing *in situ*. Presumably when whole brain tissue is homogenized with chloroform-methanol, this myelin triphosphoinositide, free or combined in a phosphatido-peptide complex as the case may be, must bind with some other macromolecule present in the non-myelinic components rendering it insoluble in the solvent. Myelin contains sufficient alkaline-earth metals to ensure that both of the monophosphoester groups of triphosphoinositide would be fully neutralized (Eichberg & Dawson, 1965).

It must be emphasized that the above arguments do not in any way indicate that triphosphoinositide exists in brain tissue as a free lipid molecule unbound to protein. Indeed with the extreme amphipathic

nature of the molecule containing both highly charged and hydrophobic regions such a situation seems extremely unlikely. Rather they cast doubt as to whether the phosphatido-peptides as isolated by solvent fractionation bear any relation to the true triphosphoinositide-protein complexes which exist in tissues.

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