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# The isolation of a new complex lipid: triphosphoinositide from ox brain. A commentary by

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on 'The isolation of a new complex lipid: triphosphoinositide from ox brain'
by J.C. Dittmer and R.M.C. Dawson
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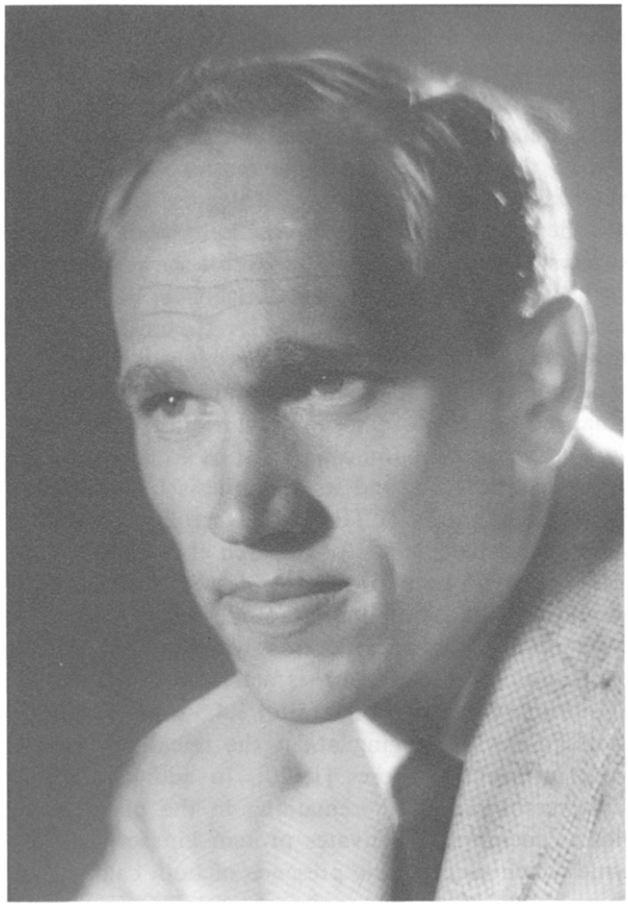
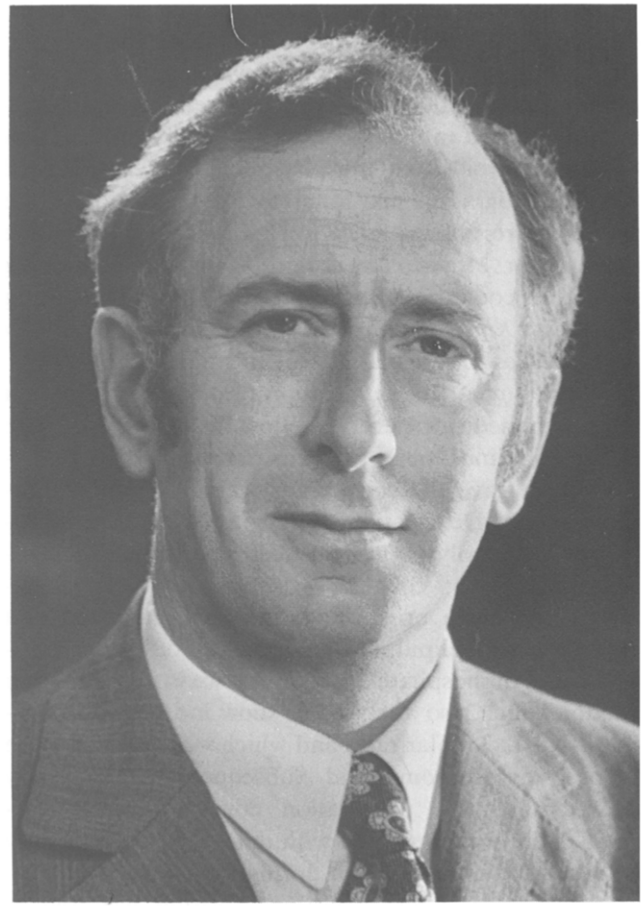
Jordi Folch's separation of the brain cephalin fraction [1] into a number of individual components remains an outstanding event in our understanding of the chemical nature of membrane phospholipids. Among the complex lipids isolated was a compound which he called diphosphoinositide containing inositol : P : glycerol and fatty acids in the molar ratios 1/2/1/1 and which liberated inositol *m*-bisphosphate after a brief acid hydrolysis [2]. Although this inositol-containing phospholipid was present in very low proportions compared to other membrane phospholipids, it rapidly became apparent that, in brain tissue at least, it had a



John Dittmer



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much more active metabolism. It was broken down at a rapid rate by brain homogenates [3] and [$^{32}$P]phosphate was rapidly incorporated into it, both in homogenates and the intact brain [3,4]. These observations elicited a great deal of interest at the time, since it strongly suggested that diphosphoinositide had a function other than the structural role which had been ascribed to the much more slowly turning over phospholipids present in membranes and multi-enzyme complexes.

Although the structure of diphosphoinositide had not been ascertained with certainty, it was decided in our laboratory that the time was opportune to prepare the pure lipid as a substrate for metabolic experiments. It was indeed fortunate that John Dittmer arrived at that time as a U.S. Public Health Service Research Fellow of the National Heart Institute. He had been well trained in lipid techniques in the laboratories of Don Hanahan in Seattle; as a pastime he owned and operated a commercial apairy containing more than two hundred hives. On attempting to isolate diphosphoinositide, it quickly became apparent that the brain phosphoinositide fraction was heterogeneous and from it were isolated two other inositol-containing phospholipids, phosphatidylinositol and a new lipid which we designated triphosphoinositide, since it contained inositol and phosphate in the molar ratio of 1 : 3.

Advances in science are generally the result of the development and introduction of new techniques, and our studies were no exception. It was possible to monitor the fractionation procedure by methods which had been developed in our laboratory whereby mild acid or alkaline hydrolysis of the phospholipids was followed by chromatographic and ionophoretic separation of the partial hydrolysis products [6,7]. In addition, inositide fractions or hitherto unrecognized inositol-containing derivatives could be precisely analysed for inositol using the yeast *Kloeckera brevis*, whose growth is totally dependent on the availability of this polyol [8].

At the time of the publication of our paper, research had progressed sufficiently to allow the isolation of pure phosphatidylinositol from brain tissue and also a fraction which gave the correct basic analysis for triphosphoinositide. Deacylation of the latter fraction by alkaline methanolysis gave glycerophosphoinositol diphosphate. This, coupled with the isolation of diacylglycerol on a brief acid hydrolysis, indicated that the new phospholipid had the structure of a phosphatidylinositol diphosphate. In addition, we isolated another fraction which also analysed to show inositol and phosphate in a 1 : 3 molar ratio and which was deduced to be a closely related compound. Subsequent studies clearly showed that this preparation consisted of triphosphoinositide contaminated with diacylglycerol probably produced by its breakdown during the isolation [9].

It was clear that triphosphoinositide was tightly bound to brain proteins; indeed, the isolation of such a protein complex followed by its disruption with denaturing solvents had been an important step in the method involved. However, at the time no information was gained as to the relation of this to Folch's original diphosphoinositide fraction. The isolation of inositol trisphosphate from such fractions after an acid hydrolysis had indicated that they contained triphosphoinositide [10], but more detailed examination of the alkaline and acid hydrolysis products showed that a true diphosphoinositide, most likely with the phosphatidylinositol phosphate structure, was also present [11].

The isolation of triphosphoinositide and the realisation that three distinct phosphoinositides existed in the brain quickly led to an ascertainment of their precise chemical structures and interrelationships [12]. The precise structure of triphosphoinositide was determined as 1-phosphatidyl L-*myo*-inositol 4,5-bisphosphate. It was biosynthesized by a successive step-wise phosphorylation of phosphatidylinositol in the 4 and 5 positions of the inositol ring [13]. Degradation was on the one hand by a specific phosphomonoesterase [14], but a phosphodiesterase liberating diacylglycerol and inositol 1,4,5-trisphosphate was also active [15]. Of course, at the time the enormous physiological importance of this latter enzyme was not realized.

Researchers had realized right from the pioneering studies of the Hokins [16] that the turnover of the phosphoinositide fraction of cells was dramatically responsive to any stimulation of its receptors which involved an internal mobilisation of calcium [17]. A vast literature accrued and for many years it was believed that in the metabolism of the most prominent phosphoinositide concentration-wise, phosphatidylinositol was the primary response to stimulation, but no clue as to physiological reason and nature of the response was forthcoming. Seven years ago Michell et al. [18] proposed that the agonist-occupied receptor activated a phosphoinositidase whose primary substrate was triphosphoinositide rather than phosphatidylinositol. The enhanced turnover of phosphatidylinositol observed was brought about by its phosphorylation to produce more triphosphoinositide, and its resynthesis by the normal metabolic channels (CTP-diacylglycerol and inositol). Since then, an explosion of research effort has revealed that the hydrolysis products released from triphosphoinositide have both important messenger roles in the cascade of biochemical events which occur when a calcium-mobilising receptor is activated by an agonist. The primary function of inositol 1,4,5-triphosphate appears to lie in bringing about the release of calcium from intracellular stores [19,20]. In addition, the diacylglycerol produced, remaining in the plane of the plasma membrane, activates protein kinase C, an enzyme which requires the presence of both calcium and phosphatidylserine as cofactor [21,22]. Thus, the breakdown of triphosphoinositide located at or near the

receptor sites in the plasma membrane produces a remarkable bifurcated signalling pathway, the versatility of which has been adapted by the cell to control cellular responses such as contraction, secretion and cell division.

Undoubtedly a great deal more remains to be discovered about the unique role which triphosphoinositide plays in cell signalling processes. Inositol 1,4,5-trisphosphate can be metabolised by phosphorylation or dephosphorylation into a considerable number of phosphorylated derivatives of inositol [23]. Some of these pathways are certainly connected with signalling suppression, yet others may influence and control other processes such as those responsible for the influx of external calcium into the cell on stimulation. The diacylglycerol liberated, as well as activating protein kinase C, may have unique physiochemical effects on the plasma membrane and influence the activity of intramembranous enzymes such as phospholipases [24]. Any arachidonic acid liberated by enhanced phospholipase activity or through the subsequent metabolism of the released diacylglycerol would in turn set up a whole series of additional responses through synthesis of lipoidal cell messengers such as the prostaglandins and other eicosanoids

The whole question of the special and temporal aspects of receptor action and the way the triphosphoinositide phosphodiesterase is activated by the presence of agonist, and the likely role of G proteins in the process, remains to be unravelled. It is clear, as well, that triphosphoinositide may have other functions apart from its role as an agonist-sensitive pool in the plasma membrane [25]. In this respect its very high concentration in the rather unusual type of plasma membrane, the rolled-up myelin-sheath formed by brain oligolendria cells, may have as yet unknown functional significance.

Thus, triphosphoinositide has proved to be a membrane constituent of central importance in many cell processes. When John Dittmer first isolated and identified this phospholipid in our laboratory, we of course had no idea of the unique role it was playing in the cellular economy. Unfortunately, due to his tragic and premature death (1974), he did not live to see the dramatic developments which have occurred in our understanding of the role of this phospholipid in the last decade. The identification of this lipid reported briefly in the BBA paper represents a significant event in the stepwise progression that science makes in unravelling the mysteries of biological phenomena and serves as a fitting scientific memorial to his talents.

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## The isolation of a new complex lipid: triphosphoinositide from ox brain

By solvent fractionation FOLCH[1] prepared from ox brain a phospholipid which he designated diphosphoinositide and which consisted in its simplest composition of one molecule each of inositol, glycerol and fatty acid together with two molecules of phosphoric acid. After short acid hydrolysis its main phosphorus-containing product was found to be inositol-*m*-diphosphate. Recently a similar diphosphoinositide fraction from brain was shown to yield three spots on a paper chromatogram[2].

FOLCH AND LEBARON[3–5] also described a complex inositol-containing phosphatido-peptide which was extracted from lipid-free trypsin-digested brain material with acidified solvent. This contained phosphorus and inositol in the molar ratio 2.7:1, a sphingosine-like substance, fatty acid, and abundant nitrogen, mainly in the form of peptide-linked amino acids; it yielded inositol diphosphate on acid hydrolysis.

Recently a new hydrolytic technique[6] has been developed for the quantitative examination of the phospholipids in a complex lipid fraction isolated from tissues. When this method was applied to lipid extracted from brain tissue by chloroform–methanol (2:1, v/v) it failed unexpectedly to give evidence for the presence of any inositol-containing phospholipid apart from monophosphoinositide. The reverse was true, however, if the tissue was pre-treated with acetone prior to the chloroform-methanol extraction. This observation has been made the basis of a method for isolating the complex inositide fraction from ox brain, the purification being followed by preparing acid and alkaline hydrolysates of the fractions and examining these by high-voltage ionophoresis and chromatography.

The brain tissue is exhaustively extracted with chloroform–methanol to remove all other lipids, and the residue then extracted with slightly acidified chloroform–methanol solvent[4]. When the latter extract is shaken with aq. 0.9 % NaCl and centrifuged, the inositide is largely found in the interfacial protein layer. After heating this with acetone and ethanol, the protein is all removed and the chloroform-soluble inositide is obtained. On adding methanol, a methanol-insoluble fraction is precipitated, designated triphosphoinositide A. If the supernatant is now neutralized with methanolic NaOH, the main component of the fraction, triphosphoinositide B, is precipitated as its sodium salt.

The sodium salt of triphosphoinositide B is soluble in water and chloroform but insoluble in methanol. It is virtually nitrogen-free. Its simplest composition is found to be $(\text{fatty acid})_6(\text{phosphate})_6(\text{glycerol})_3(\text{inositol})_2$ and this accounts for all of the components of the molecule. After brief acid hydrolysis it yields considerable diglyceride together with free glycerol, fatty acids, and a trace of free inositol. Of the water-soluble phosphorus-containing components formed, inositol triphosphate has been isolated in a yield of 75–80 %; a compound containing $(\text{inositol})_1$-$(\text{phosphate})_2$, a few per cent of glycerophosphoric acid and traces of inorganic phosphorus comprise the rest of the hydrolysate. The fatty acids on examination by gas chromatography have been shown to be a complex mixture with stearic acid, arachidonic acid and a $C_{22}$ polyunsaturated acid predominating; palmitic, oleic, linoleic acids and another $C_{20}$ unsaturated acid are also present.

A fraction equivalent in composition to the triphosphoinositide A fraction is

obtained in much better yield, by working up the lipids obtained by chloroform–methanol extraction of acetone-pretreated brain tissue. The Folch I fraction[7] prepared from such an extract is run through an Amberlite IRC 120 column which removes cations and most of the contaminating inorganic phosphate. The lipid is then distributed in a biphasic system formed from chloroform, ether, ethanol and water. The product, thus separated from contaminating phosphatidyl serine and monophosphoinositide, is insoluble in methanol. Analysis indicates the simplest composition as $(\text{glycerol})_1$-$(\text{inositol})_1$ $(\text{phosphate})_3(\text{acyl ester})_{2?}$. On brief acid hydrolysis, mono- and diglycerides can be isolated from the hydrolysate. The phosphorus-containing hydrolysis products are very similar to those from triphosphoinositide B except that more inorganic P and less inositol triphosphate are formed. After mild alkaline hydrolysis[6] the predominant phosphorus-containing product analyses as $(\text{glycerol})_1(\text{inositol})_1(\text{phosphate})_3$.

By means of alumina and silicic acid columns[8,9], monophosphoinositide giving the correct analysis has also been obtained from brain lipid extracts. This confirms the recent evidence, obtained by chromatography, for the existence of this lipid in brain tissue[2,6,9].

The present results indicate that as well as monophosphoinositide, brain tissue contains two triphosphoinositides which are probably very closely related in structure, and which are tightly attached to brain proteins. No information has yet been obtained regarding the relation of these to diphosphoinositide or the relative amounts of the various inositol-containing lipids.

Dr. D. RHODES is thanked for the fatty acid analysis of triphosphoinositide B. One of us, J. C. DITTMER, is a U.S. Public Health Service Research Fellow of the National Heart Institute.

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