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Review

A retrospective: Use of *Escherichia coli* as a vehicle to study phospholipid synthesis and function☆

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ABSTRACT

Although the study of individual phospholipids and their synthesis began in the 1920s first in plants and then mammals, it was not until the early 1960s that Eugene Kennedy using *Escherichia coli* initiated studies of bacterial phospholipid metabolism. With the base of information already available from studies of mammalian tissue, the basic blueprint of phospholipid biosynthesis in *E. coli* was worked out by the late 1960s. In 1970s and 1980s most of the enzymes responsible for phospholipid biosynthesis were purified and many of the genes encoding these enzymes were identified. By the late 1990s conditional and null mutants were available along with clones of the genes for every step of phospholipid biosynthesis. Most of these genes had been sequenced before the complete *E. coli* genome sequence was available. Strains of *E. coli* were developed in which phospholipid composition could be changed in a systematic manner while maintaining cell viability. Null mutants, strains in which phospholipid metabolism was artificially regulated, and strains synthesizing foreign lipids not found in *E. coli* have been used to this day to define specific roles for individual phospholipid. This review will trace the findings that have led to the development of *E. coli* as an excellent model system to study mechanisms underlying the synthesis and function of phospholipids that are widely applicable to other prokaryotic and eukaryotic systems. This article is part of a Special Issue entitled Phospholipids and Phospholipid Metabolism.

1. Introduction

Analysis of phospholipids and their biosynthesis were initially carried out in plants and animals and only began to focus on bacteria in the 1960s. Much of what we know about this area of research has resulted from a combination and interplay of studies in both eukaryotes and prokaryotes. My own interests in phospholipids began in 1969 as a postdoctoral fellow in Eugene Kennedy's laboratory at Harvard Medical School (Fig. 1). This was an exciting time in which the basic blue print for phospholipid biosynthesis at all levels of the genetic tree had been established and awaited the application of sophisticated methods of biochemical analysis, genetics and the power of advanced molecular biology and molecular genetics. This retrospective will be largely confined to the studies in *Escherichia coli* and their contributions to our understanding of phospholipid metabolism and function. My personal observations at various stages of the evolution of the field will be injected. I fully recognize that major contributions have been made by studies in other biological systems for which there is insufficient space to include.

2. The early studies in eukaryotic systems

2.1. Identification of phospholipids

As we now know, phosphatidic acid (PA, 1,2 diacyl-*sn*-glycerol-3-phosphate) is the precursor leading to the biosynthesis of the remaining phospholipids in *Bacteria* and *Eukarya*; see http://www.lipidmaps.org/ for classification and structures of glycerol-based phospholipids. *Archaea* phospholipids differ in that they are composed of *sn*-glycerol-1-phosphate in ether linkage at the 2- and 3-positions to long-chain poly isoprenoids [1]. The first report of the isolation and structure of PA was by Chibnall and Channon in 1927 [2,3]. They began with over 20 kg of cabbage leaves from which a

Abbreviations: ACP, acyl carrier protein; PA, phosphatidic acid; PE, phosphatidylethanolamine; P_i, PO_4; GP, glycerophosphate; PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; DAG, diacylglycerol; LPS, lipopolysaccharide; MDO, membrane derived oligosaccharide; CL, cardiolipin; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; GlcDAG, monoglucosyl diacylglycerol; GlcGlcDAG, diglucosyl diacylglycerol; PssA, phosphatidylserine synthase; PgsA, phosphatidylglycerophosphate synthase; Pgp, phosphatidylglycerophosphate phosphatase; Cds, CDP-diacylglycerol synthase; Cls, cardiolipin synthase; P_{tet}, *tet* operon promoter; IPTG, isopropyl-ß-D-thiogalactoside; P_{lacOP}, *lac* operon promotor; mAb, monoclonal antibody; TM, transmembrane domain; LacY, lactose permease; mAb, monoclonal antibody; NAO, Nonyl Acridine Orange; NBPAL, non-bilayer prone anionic lipid

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Fig. 1. The Kennedy "Clan" on the occasion of his 90th birthday. Eugene Kennedy (1919–2011) is 3rd from the left in the front row. The gathering was in October 2009 at Harvard Medical School. Pictured are former graduate students, postdoctoral fellows and scientific associates of Eugene Kennedy.

green colloidal "cytoplasmic fraction" free of cell wall material was first isolated. After heat treatment, the coagulated material was extracted with ether to yield about 35 g of "fatty" material, which was subjected to successive acetone treatments yielding a precipitate of which half by weight contained phosphorus. Further analysis revealed that the majority of the phosphorus-containing material was made up of divalent cation salts of PA and lyso-PA, which would later be shown to result from hydrolysis of the major phospholipid species.

In the early 1900s lecithin (phosphatidylcholine (PC), 1,2 diacyl-*sn*-glycerol-3-phosphocholine) was isolated from plant and egg sources and its theoretical structure proposed. However, there were some inconsistencies in several of the reports, which resulted in some controversy in the literature. Levene and West [4,5] questioned the thoroughness of previous analyses and demonstrated that the free amine containing lipidic material contained in samples of lecithin was cephalin (phosphatidylethanolamine (PE), 1,2 diacyl-*sn*-glycerol-3-phosphoethanolamine). However, by the late 1920s the natural occurrence and structures of the phospholipid precursor (PA) and the two major amino-containing phospholipids (PE and PC) were established.

2.2. Radiolabeling of phospholipids

A major technical advancement that changed the course of characterization of biological molecules in general was the availability of man-made radionuclides. The first use of $^{32}PO_4$ ($^{32}P_i$) was in 1935. Chievitz and Hevesy [6] fed $^{32}P_i$ to rats and traced radiolabel in tissue deposits and excretion. Thus it was now possible to follow lipidic phosphate fluxes in whole animals. Previous attempts to measure fluxes in phospholipids relied on feeding animals with elaidic acid [7] which falsely assumed that flux of this fatty acid through the lipidic phosphate pool was a measure of phospholipid metabolism. In 1937 Atrom et al. [8] noted that lipidic phosphate accumulated in largest amounts in liver, intestine and kidney of rats fed $^{32}P_i$. This 'organification' of phosphorus was the first proof that phospholipids were synthesized endogenously from individual building blocks. Perlman and Chaikoff [9] followed with a series of experiments that measured incorporation and turnover of $^{32}P_i$ in phospholipids in various organs either from endogenous or fed supplies of fat. In the methods section of the above paper the authors noted an important point, still missed today in many radioisotope experiments, to add unlabeled carrier phospholipid to samples with low levels of total lipid. In subsequent experiments the flux of $^{32}P_i$ through the tissue pools of phospholipid was measured as a function of dietary supplements, but only lipidic phosphate was measured and not individual phospholipids. However, incorporation of $^{32}P_i$ into phospholipid was demonstrated after incubation with homogenized liver, which set up future studies for *in vitro* incorporation experiments. It was not until the 1950s with methods available for subcellular fractionation that specific lipids were identified in organelles such as the mitochondria. Swanson and Artom [10] established that the major lipidic phosphate of rat mitochondria was composed of PE and PC.

2.3. Employing cell free systems

Arthur Kornberg [11] and Eugene Kennedy [12], using soluble enzyme extracts of tissues or mitochondria, respectively, carried out the first experiments that began to delineate phospholipid biosynthetic pathways. The hallmark of Kornberg's approach to employ enzyme preparations (not yet homogeneous as he later required) to study biosynthetic processes was evident in this early work. He used ATP, CoA and fatty acids or substituted the latter with enzymatically-synthesized palmitoyl-CoA plus enzymatically synthesized *sn*-glycerol-3-[^{32}P] (GP) to synthesize radiolabeled PA using a cell free system derived from guinea pig liver. Longer fatty acid chains (16–18) were better substrates than shorter chains. Although PA had been identified in high content in previous studies, it was now clear that the conditions used previously to process the source material generated PA from PC and PE by hydrolysis. The same was true of lyso-PA, which was not observed by Kornberg but was most likely an intermediate in PA synthesis. However, whether PA is a precursor to the amine-containing phospholipids remained to be determined. Kennedy's approach was somewhat different but the final conclusions were the same. He used isolated rat liver mitochondria incubated initially with $^{32}P_i$ and then with [^{32}P]GP with the appropriate controls to demonstrate the formation of PA, which was dependent on oxidative phosphorylation.

The literature on the synthesis of PC and PE became a bit confusing after the initial reports of Kornberg and Kennedy. Kornberg [13]