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Polyunsaturated fatty acids stimulate phosphatidylcholine synthesis in PC12 cells

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Abstract

The synthesis of phospholipids in mammalian cells is regulated by the availability of three critical precursor pools: those of choline, cytidine triphosphate and diacylglycerol. Diacylglycerols containing polyunsaturated fatty acids (PUFAs) apparently are preferentially utilized for phosphatide synthesis. PUFAs are known to play an important role in the development and function of mammalian brains. We therefore studied the effects of unsaturated, monounsaturated and polyunsaturated fatty acids on the overall rates of phospholipid biosynthesis in PC12 rat pheochromocytoma cells. Docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6) all significantly stimulated the incorporation of ^{14}C-choline into total cellular phospholipids. In contrast, monounsaturated oleic acid (OA) and the unsaturated palmitic (PA) and stearic (SA) acids did not have this effect. The action of DHA was concentration-dependent between 5 and 50 μM; it became statistically significant by 3 hrs after DHA treatment and then increased over the ensuing 3 hours. DHA was preferentially incorporated into phosphatidylethanolamine (PE) and phosphatidylserine (PS), while AA predominated in phosphatidylcholine (PC).

Keywords

membrane metabolism; phospholipids; docosahexaenoic acid; eicosapentaenoic acid; arachidonic acid; PC12 cells

1. Introduction

Polyunsaturated fatty acids (PUFAs) are constituents of the phospholipids of mammalian cell membranes, and as such are vitally important in the normal development and optimal functioning of the brain (for reviews, see [1–4]). Vertebrates cannot synthesize PUFAs de novo but rather derive them from metabolic precursor molecules of plant origin. Alternatively, long chain PUFAs (LCPUFAs) with 20 or more carbons can be obtained directly from the animal, and particularly the fish, components of the diet. The membrane phospholipids in brain contain high concentrations of the LCPUFA docosahexaenoic acid (DHA) [5], with highest levels observed in synaptic membranes [6,7], the growth cones of neurites [8,9] and rod outer segments of the retina [10,11]. Clinical and laboratory studies indicate that a number of neurodegenerative diseases involve cell membrane abnormalities which are associated with

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DHA deficiency [12–15], and that supplementation with DHA may offer protection against neuronal damage and cell death [16–20].

We recently reported in vivo studies showing that repeated administration of DHA can increase brain levels of phosphatides and of certain synaptic proteins, and that these responses are enhanced if animals also receive choline and uridine monophosphate, which raises brain CTP concentrations [21]. In order to characterize the effects of various PUFAs on phospholipid biosynthesis we have used in the present studies undifferentiated, non-transfected PC12 cells. These cells are derived from a rat pheochromocytoma [22] and have been widely used as a model system for studies of neuronal cell metabolism [23–25].

2. Materials and Methods

Cell culture

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Powdered nutrient medium DMEM and fetal bovine serum were purchased from Invitrogen (Grand Island, NY, USA) and tissue culture plastic ware from Becton Dickinson (Franklin Lakes, NJ, USA). Fatty acids were obtained from Matreya (Pleasant Gap, PA, USA). Stock solutions (10–300 mM) were prepared in ethanol, aliquoted into brown glass vials, flushed with N_2, capped, and frozen at −20°C. Each aliquot was used only once.

Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere of 95% air, 5% CO_2 in DMEM supplemented with 10% fetal bovine serum; the medium was replaced at 2-day intervals. For experiments, cells from a homogeneous pool were plated on replicate 35-mm diameter dishes and grown to subconfluence before use. At the start of an experiment nutrient medium was aspirated and the cells were washed twice with warm, sterile PBS. The cells were then incubated for 4 hrs, unless otherwise stated, in 1.5 ml/dish of serum-free DMEM which contained fatty acids at the concentrations indicated as a 4:1 molar complex with fatty acid-free BSA (100 mg/ml). Control cells were exposed to BSA only.

Phospholipid measurements

Phosphatidylcholine synthesis was measured as the incorporation into cell phospholipids of ^{14}C-choline during the final 2 hrs of the standard 4 hr incubation. At the start of this interval the medium was changed to serum- and choline-free DMEM supplemented with 10μM choline, 3 μCi/ml ^{14}C-choline (GE Healthcare, Piscataway, NJ, USA), and the fatty acids being tested. At the end of incubation the medium was aspirated, the cells were washed twice with cold PBS, and scraped into 1 ml of cold methanol. The cells were then sonically disrupted; 100 μl aliquots of the cell suspension were removed for measurement of DNA by the fluorimetric method of Labarca and Paigen [26] using calf thymus DNA as the standard. Phospholipids in the remainder of the cell sonicates were extracted by the sequential addition, with vortexing, of 1.8 ml of cold chloroform and 0.9 ml cold water [27]. The aqueous and organic phases were separated by centrifugation for 15 min. at 3000 rpm. The lower, organic phases were transferred quantitatively to borosilicate tubes, and a 50 μl aliquot of each was counted by liquid scintillation spectrophotometry as a measure of the synthesis of choline-containing phospholipids. The remainder was dried in a rotary evaporator. Individual phospholipids in the dried organic phase were separated by thin layer chromatography on silica gel G plates (Alltech, Deerfield, IL) with a mobile phase of chloroform/ethanol/triethylamine/water (30:34:30:8, by vol.). They were then identified by comparison with authentic standard preparations after spraying the plate with diphenylhexatriene in petroleum ether, and were visualized by long-wave UV light. Individual spots were scraped from the plate and quantitated by phosphate assay [28]. During a 2-hr ^{14}C-choline pulse >95% of the radioactive phospholipid fraction was

PC, Sphingomyelin contributed < 0.5%. We therefore took the incorporation of ^{14}C-choline into cell PL as a measure of PC synthesis.

Incorporation of DHA and AA into various phospholipid species was studied by incubating separate groups of dishes in serum-free DMEM containing 30 μM choline and ^{14}C-DHA (American Radiolabeled Chemicals, St. Louis, MO, USA, 0.1 mCi/ml) plus unlabeled DHA at a final concentration of 10 μM, or ^{3}H-AA (Perkin-Elmer Lifescience, Boston, MA, 0.1 mCi/ml) plus unlabeled AA at a final concentration of 10 μM. Extraction, separation and identification of individual phospholipids species was performed as described above. Spots corresponding to PC, PS, PI, and PE were scraped into scintillation vials, eluted with 500 μl methanol and the radioactivity was measured by liquid scintillation spectrophotometry.

Data Analysis

Results were normalized with respect to cell DNA and expressed as means ± SEM of measurements from sextuplicate or quintuplicate dishes. Differences between means were analyzed by ANOVA, followed by Student's *t* test for the comparison of two means or by Dunnett's test for the comparison between multiple means and their control. Differences were accepted as significant at $p<0.05$.

3. Results

Fatty acids and phospholipid synthesis

Incubation of PC12 cells with the unsaturated palmitic acid or the monounsaturated oleic acid, both at 20 μM concentration, did not stimulate ^{14}C-choline incorporation into cellular PL, while incubation with LCPUFAs AA and DHA were stimulatory (Fig 1A). In separate experiments the saturated fatty acid stearic acid (20 μM) failed to stimulate PL synthesis, while EPA, an LCPUFA, at the same concentration, was stimulatory (Fig.1B).

In order to ascertain that the observed effect of PUFAs was not due to inhibition of PL breakdown, PC12 cells were incubated in the presence of 20μM DHA and the concentration of unlabeled PC was measured. DHA supplementation was indeed found to increase cellular PC levels significantly (Fig.2). This response was concentration-related between 5 and 50 μM DHA (Fig.3). Concentrations greater than 100 μM caused numbers of cells to become detached from the dishes during incubation.

Time course of DHA effect

The accumulation of PL in PC12 cells with time after the addition of 20 μM DHA to the medium is shown in Fig. 4. Because the rates of synthesis during the first hours of incubation were variable for both control and treated cells, results are expressed as ratios of labeled PL in DHA treated/control cells (dpm/μg DNA). Between 2 and 6 hours of incubation, newly synthesized PL accumulated in nearly linear fashion to a significantly greater extent in DHA-treated cells than in the corresponding controls. During these incubation periods in serum-free medium cell viability was maintained as judged by cell attachment or by Trypan Blue exclusion.

Incorporation of AA and DHA into phospholipids

Parallel groups of dishes were incubated with either ^{3}H-AA or ^{14}C-DHA. Because the specific activity of the radiolabeled molecules was low, the concentrations of unlabeled PUFAs added to the incubation medium were reduced to a final concentration of 10 μM. The concentrations of individual phospholipid esters were determined by phosphate assay from a third group of dishes and the specific activities of radiolabeled AA and DHA incorporated into each were calculated. DHA was preferentially incorporated into PS and PE, while AA incorporation predominated in PC. PI synthesis utilized both DHA and AA to a comparable extent (Fig.5).

4. Discussion

The results presented here show that increasing the availability of such LCPUFAs as AA, EPA and DHA can increase both the synthesis (Figs. 1A and 1B) and levels (Fig. 2) of PC in undifferentiated PC12 cells. This effect is time- and concentration-dependent, and is specific for PUFAs; monounsaturated and saturated FA were ineffective. In contrast to our findings, Pelech et al. observed significant stimulation by oleic acid as well as by PUFAs of choline incorporation into phosphatidylcholine in HeLa cells [29]. This discrepancy may reflect inherent differences between human and rat cells, or perhaps differences in the concentrations of fatty acids used in the two studies (i.e. 1 mM OA cf. our 20 μM).

Previous studies have shown that other circulating PC precursors, specifically choline and cytidine or uridine, also stimulate PC synthesis in slice preparations of rat striatum [30] or in PC12 cells [24] by combining to form CDP-choline, one of the two proximate precursors of PC synthesis [31]. The final step in PC synthesis, catalyzed by CDP choline:1,2-diacylglycerol phosphotransferase [32], attaches CDP-choline to diacylglycerol (DAG). Under conditions where NGF-stimulated neurite outgrowth required new membrane formation, increased availability of DAG also was found to stimulate PC synthesis [33,34]. Because these effects of NGF would confound any stimulation of PC synthesis that might have been caused by PUFAs, our experiments used undifferentiated PC12 cells in defined medium. When gerbils received uridine in the diet and DHA by gavage, significant increases in brain phospholipids and in specific synaptic proteins were observed, while the increases caused by uridine or DHA alone were less pronounced [21].

Due to their hydrophobic nature PUFAs cross the blood/brain barrier and enter membrane-delimited compartments with relative ease, aided by intramembranous long-chain fatty acid transport proteins [35]. In order to sustain the observed intracellular accumulation of LCPUFAs in brain cells, retrograde repartitioning is prevented by the binding of PUFAs to fatty acid binding proteins, followed by their rapid conversion to fatty acid coenzyme A (CoA) derivatives [36]. Kinetic studies of FA uptake by a variety of mammalian cell preparations revealed a saturable process with K_m values in the nM range [36,39–41]. This propensity to be saturated at low ligand concentrations necessarily limits intracellular concentrations of FAs. Following their uptake and translocation across the cell membrane the FAs are activated by a family of ACSs, each with particular preferences for various unsaturated, monounsaturated or polyunsaturated FAs, and with substrate K_ms in the μM range [36–38,42–44]. It is therefore likely that the ACS enzymes responsible for activating LCPUFAs prior to their incorporation into PLs will be unsaturated and thus responsive to changes in substrate availability.

Differences in the patterns of saturated and unsaturated FAs found in particular phospholipid species have been described [10,23,45,46]. We also found that PC12 cells preferentially incorporated different PUFAs into particular phospholipid species. Thus, AA was incorporated into PC to a greater extent than was DHA, while DHA predominated in PS and, to a marked extent in PE; PI incorporated both FAs (Fig.5). These findings agree with those described in rat and beef brain preparations [47,48]. AA and DHA are incorporated into PI of PC12 cells at almost identical levels while in rat brain AA predominated over DHA by a factor of 4:1; in implanted rat brain tumor the ratio is 2.5:1 [47]. The neoplastic origin of PC12 cells may account for an AA/DHA ratio more closely related to that of tumor tissue than to normal brain.

It has been suggested that cholinephosphotransferase and ethanolaminephosphotransferase may have different substrate specificities with regard to the FA composition of diacylglycerol [45,46], while De Kruyff and his coworkers found no such specificity in rat liver microsomes [49]. The preferential labeling of PS by DHA most likely reflects the synthesis of PS from PE by base-exchange [50]; Holbrook documented a preference of the choline base-exchange

enzyme for DHA-containing phospholipids [51]. A study of rat hepatocytes incubated in vitro with fatty acids of various chain length and degrees of unsaturation showed that the composition of diacylglycerol, as well as of the PC and PE derived from it, varied depending on the fatty acid supplied in the incubation medium [46]. We therefore propose that the observed stimulation of PC synthesis in PC12 cells by DHA and other LCPUFAs results from enhanced activation of LCPUFAs by their respective, specific acyl-CoA synthetases [38], their subsequent incorporation into diacylglycerols enriched in LCPUFAs [46], and the utilization of these diacylglycerols in phospholipid synthesis.

Several neurodegenerative diseases are known or suspected to involve the breakdown of membranes, leading to cell death. Conversely, maintenance and expansion of synaptic connections requires the formation of new membrane [8,37,52]. Thus an understanding of how the dynamic requirements for membrane synthesis can be controlled may aid our ability to sustain optimal neuronal function in health and disease. We have now demonstrated that, by increasing the availability of LCPUFAs, PC synthesis and concentration are stimulated in untransfected and undifferentiated PC12 cells, even when these cells are not undergoing NGF-stimulated neurite outgrowth.

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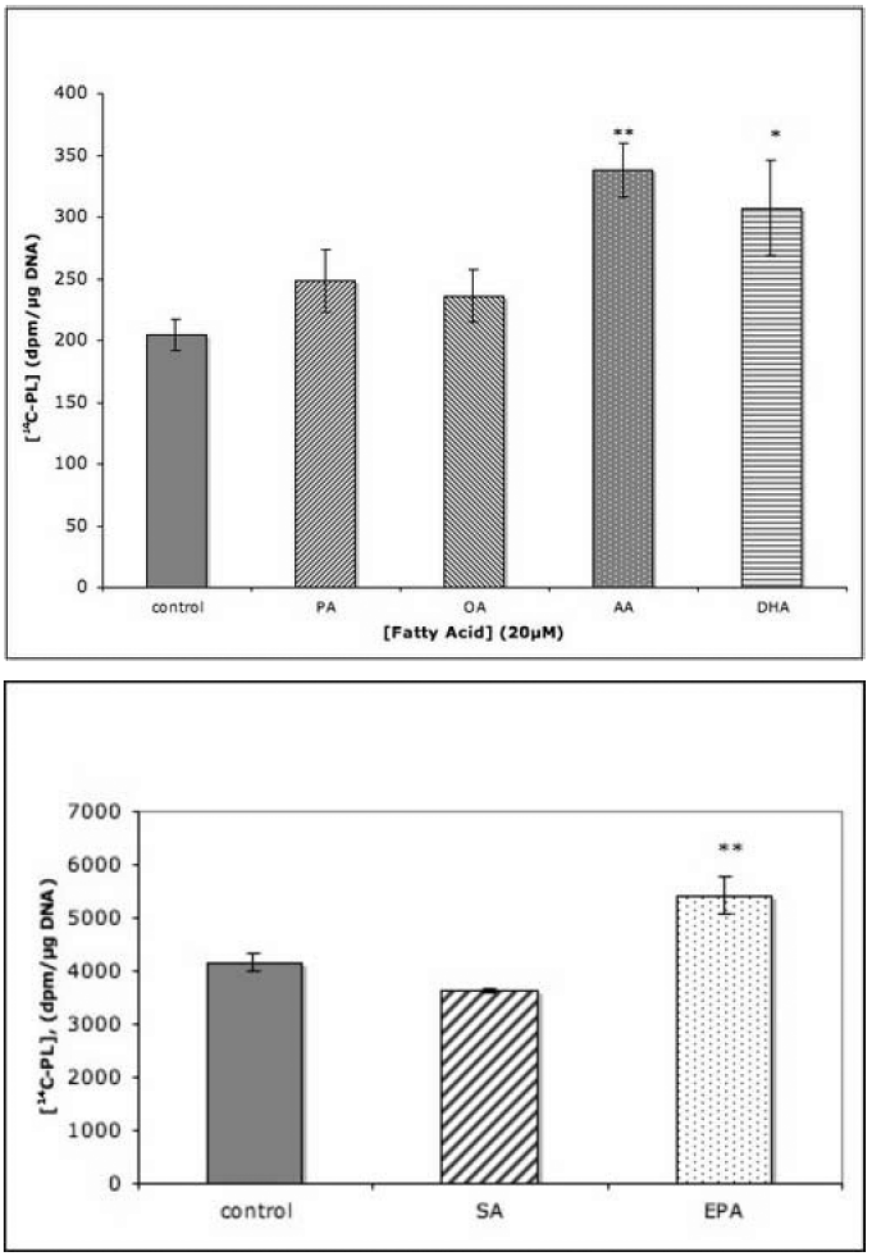

Fig 1. Effect of fatty acids on phospholipid synthesis in PC12 cells

Cells were incubated for 4 hrs with fatty acids (20 μM) and, during the last 2 hrs of incubation, with ^{14}C-choline (3 μCi/ml). Radioactivity incorporated into total cell phospholipids was measured. Data are the means ± SEM of 6 dishes and represent (A) one of 3 or (B) one of 2 independent experiments. *, P<0.05; **, P<0.01.

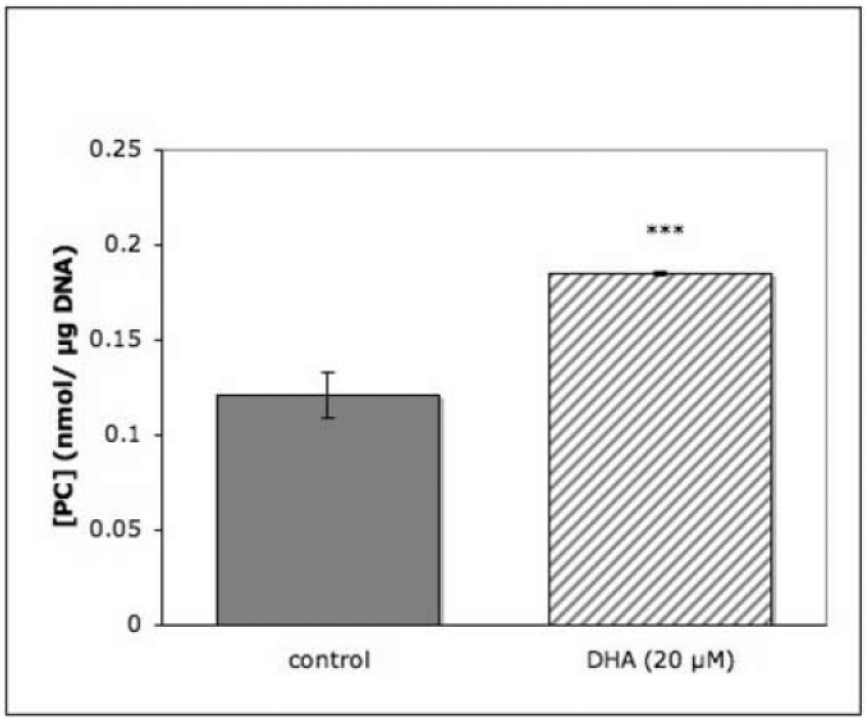

Fig 2. DHA effect on phosphatidylcholine level in PC12 cells

Replicate dishes were incubated for 4 hrs in the absence or presence of 20 μM DHA. Phospholipids were extracted, separated and quantitated as described in Experimental Procedures. Results are shown as the means ± SEM of 6 dishes from one of two experiments. ***, P<0.001.

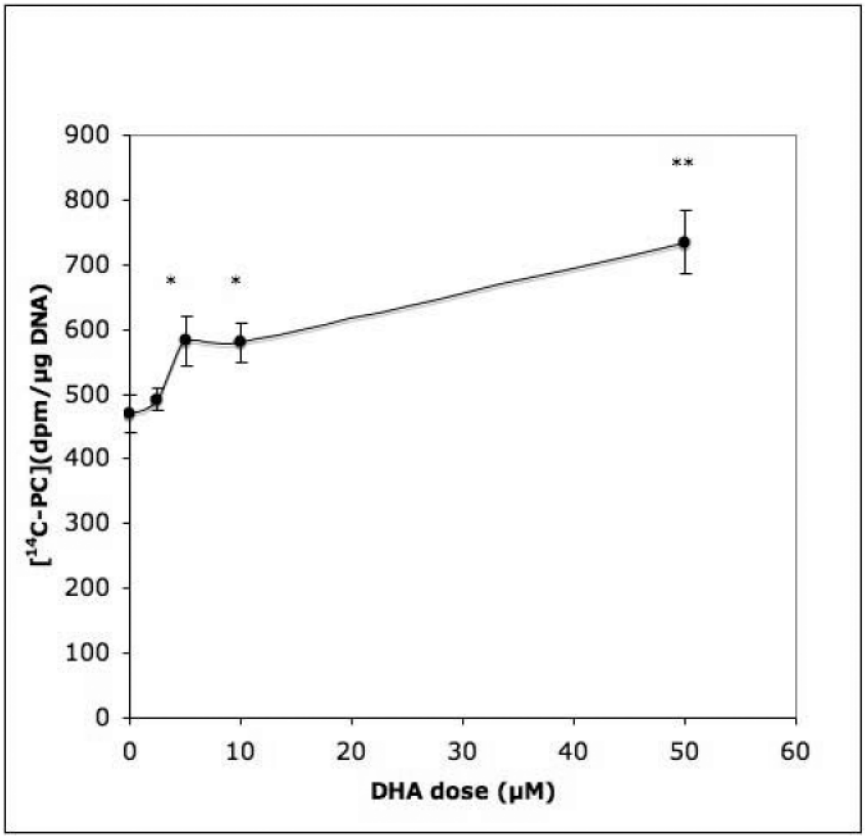

Fig 3. DHA dose and phosphatidylcholine synthesis in PC12 cells

Cells were incubated for 4 hrs with the indicated doses of DHA (2.5, 5, 10, 50 μM). Incorporation of ^{14}C-choline into total phospholipids during the last 2 hrs of incubation was measured. Data show the means ± SEM of 6 dishes, representative of 2 experiments. *, P<0.05; **, P<0.01.

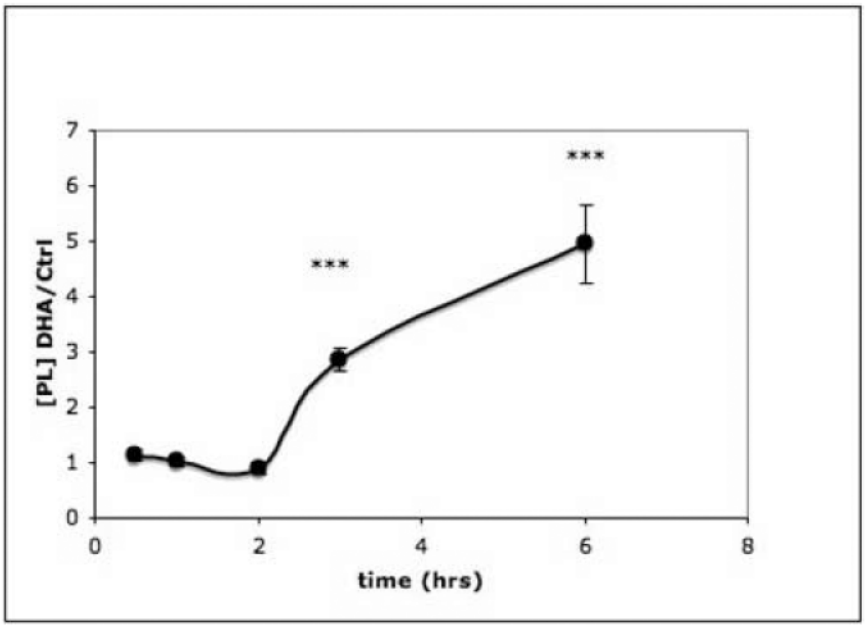

Fig 4. Time course of DHA-stimulated phospholipid synthesis

Replicate dishes of PC12 cells were incubated with or without 20 μM DHA for the time periods indicated and incorporation of ^{14}C-choline into phospholipids during a 2 hr period was measured. For incubations less than 2 hrs, the addition of ^{14}C-choline preceded addition of DHA. Results are from 6 dishes/group and are expressed as the ratio of DHA-treated/untreated cells. The measurement in each group is [^{14}C-phospholipid], (dpm/μg DNA). The experiment was repeated twice. ***, P<0.001.

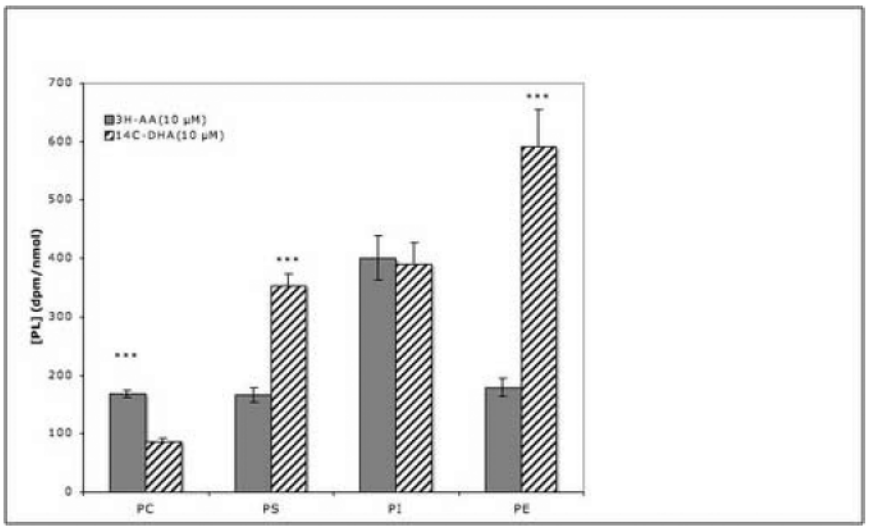

Fig 5. Incorporation of AA and DHA into phospholipid esters

Replicate dishes of PC12 cells were incubated for 3 hrs with 0.1 μCi ^{3}H-AA or ^{14}C-DHA and with 10 μM of the corresponding nonradioactive fatty acids. Phospholipids were extracted, separated by TLC and the radioactivity in individual spots was measured. Quantitites of PC, PS, PI and PE were determined by phosphate analysis. Data are the means ± SEM of 5 dishes and are expressed as dpm ^{3}H or ^{14}C incorporated/nmol PO_4. ***, P<0.001 for differences between AA and DHA-treated groups.