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Biosynthetic Labeling and Two-Color Imaging of Phospholipids in Cells

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Phospholipids with a choline head group are abundant components of all biological membranes, performing critical functions in cellular structure, metabolism, and signaling. In spite of their importance, our ability to visualize choline phospholipids in vivo remains very limited. We present a simple and robust chemical strategy to image choline phospholipids, based on the metabolic incorporation of azidocholine analogues, that accurately reflects the normal biosynthetic incorporation of choline into cellular phospholipids. Azidocholine-labeled phospholipids can be imaged in cells with high sensitivity and resolution, following derivatization with fluorophores, by bio-orthogonal chemical reactions compatible with live-cell imaging. We used this method to visualize the subcellular localization of choline phospholipids. We also demonstrate that double metabolic labeling with azidocholine and propargylcholine allows sensitive two-color imaging of choline phospholipids. Our method represents a powerful approach to directly image phospholipids, and to study their dynamics in cells and tissues.

Introduction

Choline (Cho)-containing phospholipids are present in all organisms and are the most abundant lipids in eukaryotes.[1] Aside from playing important roles in membrane structure, Cho phospholipids are critical in cell–cell signaling and as precursors of intracellular second messengers (such as arachidonic acid) or of intercellular signaling molecules (such as platelet activating factor, PAF). The main classes of Cho phospholipids are phosphatidylcholine (PC), ether phospholipids with a Cho head group (ePC), and sphingomyelin (SM). Cho is an essential nutrient for most organisms; following its cellular uptake by specific membrane transporters and its activation, it is incorporated into phospholipids in the form of the high-energy intermediate CDP-Cho.[2] Although much is known about the metabolism of Cho phospholipids, their cell biology remains obscure; this underscores the need for methods that allow them to be imaged in cells and in animals at high-resolution. In previous work,[3] we developed a strategy to image Cho phospholipids, that was based on metabolic labeling with the alkyne analogue propargylcholine (PCho), followed by detection by Cu^{I}-catalyzed alkyne–azide cycloaddition (CuAAC).[4] Although this approach is sensitive and robust, it would be desirable to have more than one bio-orthogonal method to image Cho phospholipids in vivo, particularly for dynamic experiments in which two-color detection of Cho phospholipids is required, as well as for live-cell-imaging experiments.

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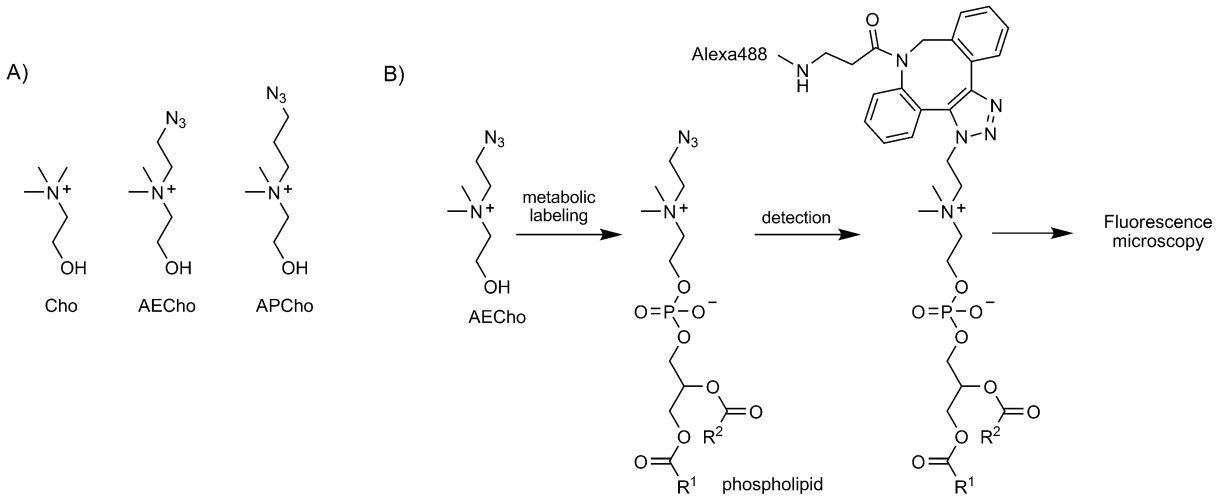
Results and Discussion

Classic studies have shown that Cho analogues in which a methyl group is replaced by an alkyl chain of up to five carbons in length are incorporated efficiently into phospholipids in insect embryos.[5] With this structure–activity relationship in mind, we synthesized two azidocholines, 1-azidoethyl-choline (AECho) and 1-azidopropyl-choline (APCho; Scheme 1 A). Our hypothesis was that at least one of these azidocholines would be metabolically incorporated into phospholipids, which could then be detected by bio-orthogonal reaction with a fluorescent reagent such as a strained cyclic alkyne[6] or a phosphine derivative[7] (see Scheme 1 B for a depiction of the detection reaction with a strained alkyne).

To determine if AECho and/or APCho could be used as Cho probes, we measured their metabolic incorporation into cellular phospholipids. Additionally, as a good Cho probe should not perturb the normal composition of Cho and non-Cho phospholipids, we also determined the effect of AECho and APCho on endogenous cellular phospholipids. Cultured mammalian cells were labeled with increasing concentrations of AECho and APCho, and total lipids were isolated and analyzed by electrospray ionization–tandem mass spectrometry (ESI-MS/MS). AECho (Figure 1 A) and APCho (Figure S1 A in the Supporting Information) did not perturb the distribution of non-Cho phospholipids, even when used at high concentration. Additionally, both AECho and APCho were tolerated well by the cells, which did not show toxicity when incubated for 24 h



Scheme 1. A) Structure of choline and the analogues, 1-azidoethyl- and 1-azidopropyl-choline (AECho and APCho). B) Metabolic incorporation of AECho into cellular phosphatidylcholine (PC). The resulting azidoethyl-phosphatidylcholine (AE-PC) is shown being detected by strain-promoted azide–alkyne cycloaddition with a fluorescent conjugate of dibenzocyclooctyne (DBCO), Alexa488–DBCO. R^1 and R^2 denote the fatty acid residues of AE-PC molecules.

with concentrations of up to 1 mM of analogue. Gratifyingly, AECho (Figure 1B) and APCho (Figure S1B) were incorporated into phospholipids in a concentration-dependent manner, without affecting the distribution of normal Cho among phospholipid classes. Consistent with the known structure–activity relationship for Cho analogues, AECho was incorporated into phospholipids much more efficiently than APCho (Table S1). At the highest concentration and after 24 h of labeling, almost 20% of the total Cho phospholipids had acquired an AECho head group. It is worth noting that we performed all labeling experiments in normal tissue culture medium, which contains 30 μM normal Cho; it is thus likely that even higher degrees of AECho labeling can be achieved under Cho-free conditions. As seen before for PCho,[3] incorporation of AECho into ePC and SM lagged behind that for PC (Figure 1B); this is likely the result of slower equilibration of AECho into the cellular pools of ePC and SM. Due to the significantly higher labeling efficiency, we selected AECho as candidate for a biosynthetic Cho probe.

To characterize AECho metabolic incorporation into phospholipids in more detail, we measured the fatty acid (FA) composition of all phospholipid species from cells incubated with varying concentrations of AECho. The sum FA composition of non-Cho phospholipids (phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidic acid) was unaffected by AECho, irrespective of concentration (Figures S2 and S3). The sum FA composition of AECho-labeled phospholipids was very similar to that of the corresponding Cho phospholipids (Figures 1C, S4, and S5). Together, these results demonstrate that AECho labels cellular phospholipids in a manner very similar to normal Cho, and without perturbing the normal composition of Cho and non-Cho phospholipids; thus AECho fulfills the stringent criteria required of a good Cho probe.

To develop a protocol for microscopic imaging of azidocholine-labeled phospholipids, we turned to two bio-orthogonal chemical reactions that have been used to detect azido compounds, strain-promoted azide–alkyne cycloaddition[6] and the Staudinger ligation.[7] In the first approach, cells labeled with AECho or APCho were treated with a fluorescent derivative of dibenzocyclooctyne (DBCO),[6c] followed by fluorescence microscopy. As shown in Figure 2A, cells labeled with AECho showed very strong fluorescent staining, with a pattern characteristic of incorporation into cellular membranes. As expected, the stain was excluded from the nucleus. At higher magnification, it became obvious that AECho-labeled phospholipids localized to the cell surface, as well as to the membrane of numerous intracellular organelles (Figure 2B). Consistent with its much lower incorporation into phospholipids, cells labeled with APCho showed weaker fluorescent staining. The staining for APCho, however, was specific (compare with the control, unlabeled cells in Figure 2A) and had the same pattern as for AECho. Thus, even very low levels of phospholipid labeling with APCho can be reliably imaged, and this indicates the high sensitivity of our microscopic method. We obtained similar results when we visualized AECho- or APCho-labeled phospholipids by using the Staudinger ligation[7b] with a biotin-phosphine derivative (Figure S6A).

The Cho head group is installed on phospholipid molecules in the endoplasmic reticulum (in the case of PC) or in the Golgi apparatus (in the case of SM); from these organelles, Cho phospholipids move to all other cellular membranes. To determine if AECho-labeled phospholipids spread similarly from their place of synthesis, we tested whether they localize to the mitochondrial membranes. Cells expressing Red Fluorescent Protein targeted to mitochondria were metabolically labeled with AECho, then stained with the Alexa488–DBCO conjugate. As shown in Figure 2B, the AECho stain showed strong co-localization with RFP, thus indicating that AECho phospholipids had been transported from the ER and Golgi to mitochondria. Thus AECho is a suitable probe for microscopy studies of intracellular phospholipid transport, a poorly understood biological process.

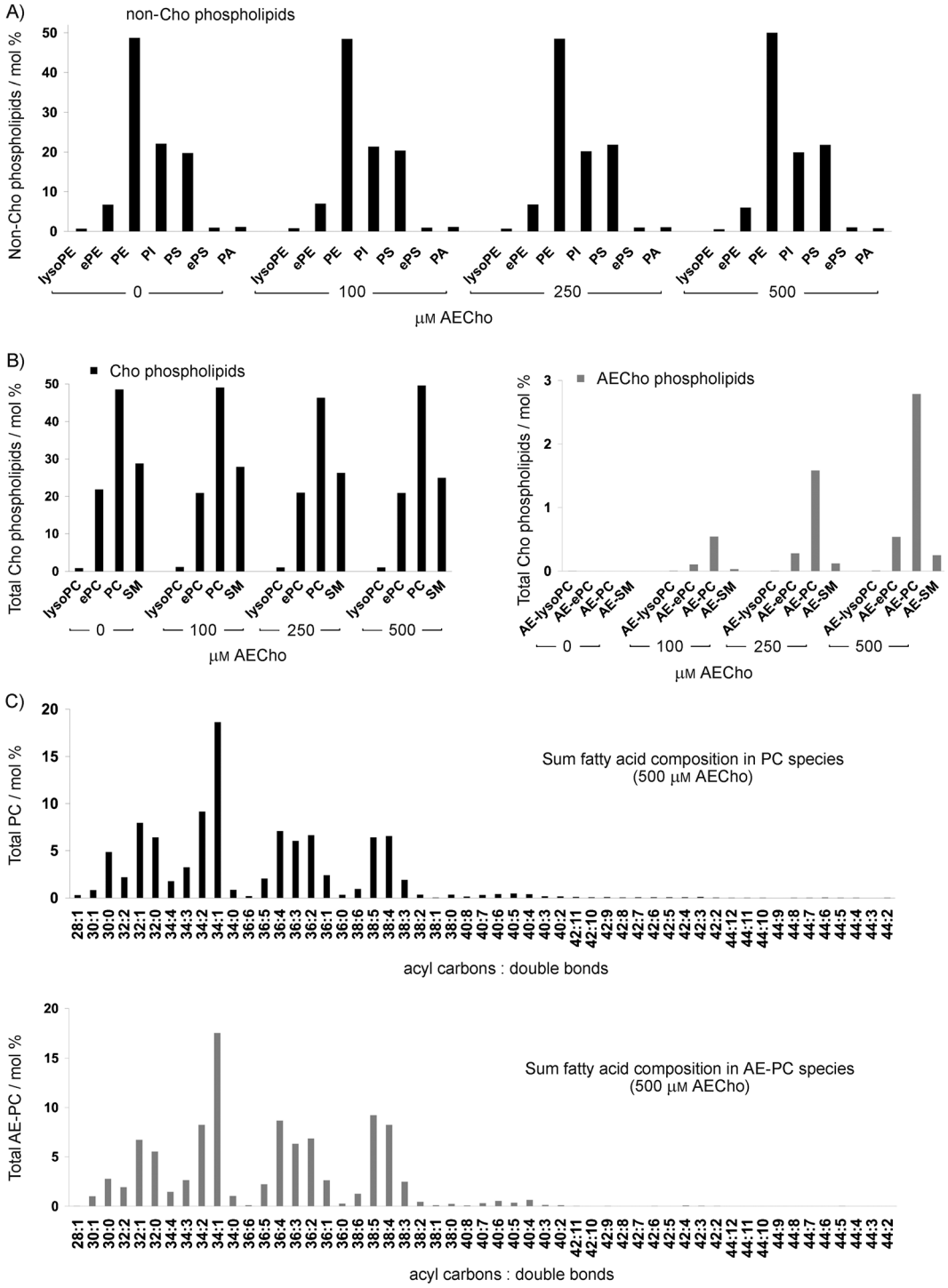


Figure 1. Metabolic labeling of phospholipids with azidocholines. A) Total lipids were isolated from NIH-3T3 cells labeled overnight with the indicated concentrations of AECho, and phospholipids were quantified by ESI-MS/MS. The distributions of lyso-phosphatidylethanolamine (lysoPE), ether-linked phosphatidylethanolamine (ePE), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) are shown as mole percentages of total non-Cho phospholipids. Metabolic labeling with AECho does not perturb the relative levels of non-Cho phospholipids. B) As in (A), but showing the distribution of phospholipids containing Cho (left) or AECho (right), as mole percentages of total Cho phospholipids. The Cho phospholipid species measured are lysophosphatidylcholine (lysoPC), ether-linked phosphatidylcholine (ePC), phosphatidylcholine (PC) and sphingomyelin (SM). AECho is incorporated in a dose-dependent manner into all Cho phospholipids. C) As in (A), but showing the sum fatty acid composition of PC species (top) and AE-PC species (bottom) in cells labeled with AECho (500 μM), as mole percentages of total PC and AE-PC. PC and AE-PC species are identified by two numbers: the sum of acyl carbons and the sum of double bonds present in the two fatty acid residues of the respective PC species. AECho incorporation into AE-PC species is very similar to that of Cho itself.

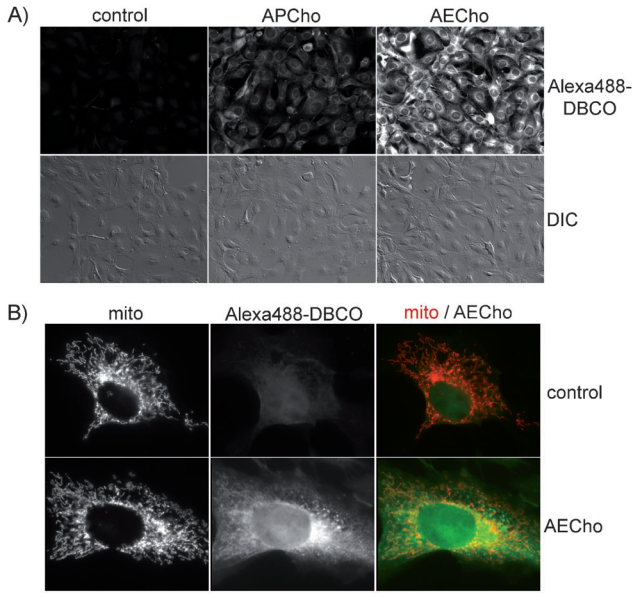


Figure 2. Imaging of cellular phospholipids labeled with azidocholines. A) NIH-3T3 cells were incubated overnight with AECho or APCho (250 μM of either analogue). The cells were then fixed, treated with Alexa488-DBCO, and imaged by epifluorescence and by differential interference contrast (DIC) microscopy. Unlabeled cells were used as negative controls. AECho- and APCho-labeled phospholipids localize to various cellular membranes, including the plasma membrane and various intracellular organelles. B) As in (A), but before labeling with AECho, the cells were transfected with pDsRed-Mito, to mark their mitochondria with Red Fluorescent Protein. AECho-labeled phospholipids show strong localization to the mitochondria.

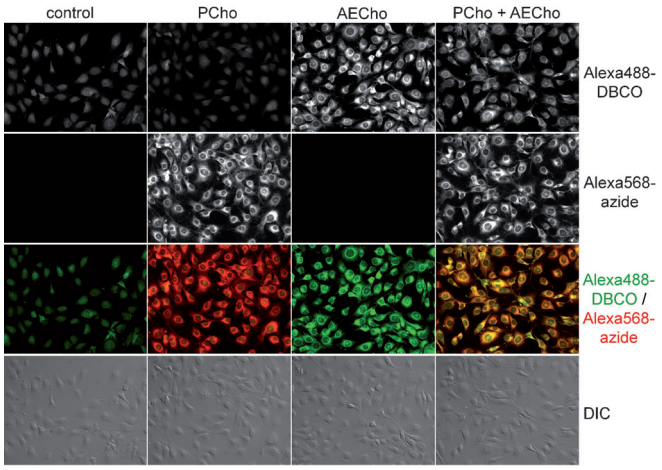


Figure 3. Two-color imaging of choline phospholipids in cells. NIH-3T3 cells were incubated overnight with control medium, PCho (100 μM), AECho (250 μM), or both. The cells were treated with Alexa488–DBCO (to detect AECho phospholipids), followed by staining by CuAAC with Alexa568–azide (to detect PCho phospholipids). Top row: AECho-labeled phospholipids; second row: PCho-labeled phospholipids; third row: overlay of the AECho and PCho images; bottom row: DIC images of the cells. Note the strong subcellular co-localization of PCho phospholipids and AECho phospholipids. Also note that Alexa488–DBCO shows a higher nonspecific background staining than Alexa568–azide, this is likely due to the hydrophobic nature of the cyclooctyne moiety.

Studies of choline phospholipid dynamics in cells would greatly benefit from the ability to label and detect two distinct phospholipid populations; however, no current methodology can accomplish this. We combined AECho and PCho labeling to develop a robust protocol for two-color imaging of cellular choline phospholipids. Briefly, cells were labeled with AECho, PCho, or both. AECho-labeled phospholipids were first treated with Alexa488–DBCO, after which PCho-labeled phospholipids were treated with Alexa568–azide by CuAAC. As shown in Figure 3, this method allowed the specific, simultaneous detection of both AECho- and PCho-labeled phospholipids. Importantly, the two populations of phospholipids had very similar subcellular distribution, and showed strong colocalization; this is consistent with the fact that both AECho and PCho are fully functional Cho analogues. We also achieved robust two-color imaging when AECho-labeled phospholipids were detected by the Staudinger ligation, followed by detection of PCho-labeled phospholipids by CuAAC (Figure S6B).

In summary, we have developed a new chemical method to directly image Cho phospholipids in cells that is based on AECho, an azido analogue that faithfully mimics Cho. AECho is efficiently incorporated into all classes of Cho phospholipids, and AECho-labeled molecules can be detected with high sensitivity by bio-orthogonal reactions with fluorescent reagents. Uniquely, double metabolic labeling with AECho and PCho can be used for two-color imaging of cellular phospholipids. We anticipate that our approach will be particularly useful for dissecting phospholipid dynamics in cells and tissues.

Experimental Section

Mouse NIH-3T3 cells were labeled overnight with AECho or APCho iodide in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% bovine calf serum, penicillin and streptomycin (complete media). After labeling, the cells were washed and then fixed with 3.7% paraformaldehyde in PBS, followed by fluorescent staining. For staining by strain-promoted azide–alkyne cycloaddition, the cells were incubated with Alexa488–DBCO (20 μM in Tris-buffered saline (TBS)), for 30 min at room temperature, after which unreacted Alexa488–DBCO was removed by washing with TBS. For staining by Staudinger ligation, the cells were incubated with biotin-phosphine (20 μM in TBS with 1 mM dithiothreitol), for 3 h at room temperature. After unreacted biotin-phosphine had been washed away, the cells were stained with Alexa488–streptavidin (1 $\mu g\,mL^{-1}$ in TBS + 4% bovine serum albumin). Finally, the cells were imaged by epifluorescence microscopy and by DIC. Images were collected by using Metamorph image acquisition software (Applied Precision) with identical camera settings for each condition.

To image phospholipids localized to mitochondria, NIH-3T3 cells were transiently transfected with pDsRed-Mito (Clontech), which labels mitochondria with Red Fluorescent Protein. The cells were labeled overnight with AECho, followed by staining with Alexa488–DBCO.

For simultaneous detection of AECho- and PCho-labeled phospholipids, NIH-3T3 cells were labeled overnight with AECho (250 μM) and/or PCho (100 μM). The cells were first treated with Alexa488–DBCO, followed by PCho detection via copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) with Alexa568–azide (10 μM), as previously described.[3]

For lipid profiling by ESI-MS/MS, total lipids were isolated from labeled NIH-3T3 cells by methanol/chloroform (2:1) extraction.[8] Au-

tomated ESI-MS/MS, data acquisition and analysis, and acyl group identification were performed as previously described,[3,9] with modifications (Supporting Information). To determine the absolute amount of various phospholipid classes, the total lipid extract was combined with internal standards, and quantified as previously described.[10] Phospholipid species containing a given head group were detected from spectra obtained by sequential precursor and neutral loss scans of the total lipid extracts.[3]

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