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ω10-Lipoxygenase products of α-linolenic acid are esterified to phospholipids in *Hydra vulgaris*

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Abstract. We have recently reported the occurrence of 9(*R*)-hydroxy-, 9(*R*)-hydroperoxy- and 9-keto-octadeca-10*E*,12*Z*,15*Z*-trienoic acids (9-HOTrE, 9-HPOTrE and 9-KOTrE) in *Hydra vulgaris*, and their biosynthesis from α-linolenic acid (α-LA) through the action of an enantioselective ω10(*R*)-lipoxygenase (ω10-LO). Here we describe the finding of these α-LA metabolites as esters to the 2-position of phosphatidylcholine, phosphatidylethanolamine and, in trace amounts, phosphatidylinositol. Small amounts of a compound co-eluting with an authentic standard of 9(*R*)-hydroxy-octadeca-10*E*,12*Z*-dienoic acid, a metabolite potentially derived from the action of ω10-LO on linoleic acid, were also found esterified with phospholipids. Since direct peroxidation of membrane lipids has been described, experiments were aimed at establishing whether α-LA metabolite-containing phospholipids could originate, in *H. vulgaris*, from either spontaneous or ω10-LO-catalyzed oxidation of phospholipid-bound α-LA. Incubation of either unlabelled or radiolabelled PUFA-containing phosphoglycerides with *H. vulgaris* ω10-LO did not result in their peroxidation. This suggests that α-LA and LA metabolites are incorporated into glycerophospholipids after their formation by ω10-LO, and that, as in mammals, membrane phospholipids may serve as a reservoir for these bioactive compounds. This is the first example in an invertebrate species of lipoxygenase products esterified to phosphoglycerides.
Key words. Hydroxyoctadecatrienoic acids; polyunsaturated fatty acids; lipoxygenases; invertebrate oxylipins; *Hydra*; HETEs.

The hydrozoan species belonging to the genus *Hydra* have, since the eighteenth century, been widely used as models for studies of several aspects of cell development[1]. Lately, these coelenterates have also been proposed as a model for the study of various long-term responses (head and tentacle regeneration, ectopic head and bud formation, larval metamorphosis) induced by lipid mediators such as protein kinase C (PKC) activators (i.e. phorbol esters and diacylglycerols), polyunsaturated fatty acids (PUFAs) and their metabolites, lysophospholipids, etc.[2–15].

Recently, both marine and freshwater hydrozoans have also been utilized as a model in investigations of the oxidative metabolism of AA and other PUFAs. Polyps from these coelenterates have been found to contain abundant, regiospecific and enantioselective lipoxygenase (LO) activities leading to the hydroperoxidation of AA with subsequent formation of bioactive hydroperoxy- and hydroxy-derivatives. In *Hydractinia*, a 8(*R*)-LO is responsible for the production of 8(*R*)-hydroxy-eicosatetraenoic acid (8(*R*)-HETE) and, possibly, of metabolites involved in the control of metamorphosis[7]. In *H. magnipapillata*, two distinct LOs catalyse the synthesis of 12(*S*)-HETE and 11(*R*)-HETE, which influence, respectively, DAG-induced bud production and ectopic head formation[16]. In the marine species *Halochordile disticha* and *Aglaophenia pluma*, low amounts of metabolites with chromatographic behaviours identical to those of the LO products 11-HETE and 9-HETE were found[17]. Finally, *H. vulgaris* was quite unique in containing a rare LO activity capable of catalysing the *R*-peroxidation of the ω10 carbon atom of C_{18}, C_{20} and C_{22} PUFAs, thereby producing metabolites that, in some cases, were active in enhancing the average tentacle number of excised regenerating polyps[18,19]. The natural substrate for this ω10-(*R*)-LO was α-linolenic acid, which is the most abundant PUFA present in *H. vulgaris* (both as free acid and esterified to the 2-position of phosphoglycerides), and, therefore, serves as the precursor of the only LO products found in measurable amounts in this hydrozoan, i.e. 9(*R*)-hydroperoxy-, 9(*R*)-hydroxy- and 9-keto-octadeca-10*E*,12*Z*,15*Z*-trienoic acids (9-HOTrE, 9-HPOTrE and 9-KOTrE, ref. 20).

Incorporation of hydroxy-fatty acids into phospholipids and triglycerides was first shown in polymorphonuclear neutrophils[21], and subsequently this finding was extended to a wide range of cell types (for review see ref. 22). Although a role in phosphatidylinositol (PI)-mediated transmembrane signalling has been proposed[23], the precise physiological significance of this kind of remod-

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eling of membrane phospholipids is still far from being clear. It has been also hypothesized that HETE esterification to the 2-position of phosphoglycerides is used by cells simply as a means to store physiologically active eicosanoids, formed in excess during previous cell stimulation, and ready to be released upon stimulation of PLA_2 and, eventually, to be metabolized further. This hypothesis found support in a study showing how PI-bound 5- and 15-HETE could be mobilized in polymorphonuclear neutrophils stimulated with the calcium ionophore, A23187 and by phorbol esters, and subsequently oxidized to di-hydroxy-fatty acids and, in the case of 15-HETE, to lipoxins[24]. In a more recent investigation, specific incorporation of 15(*S*)-HETE into PI seemed to parallel the PKC-dependent supersensitivity of the β-adrenergic response induced by this oxygenated fatty acid[25]; 12(*S*)-HETE, which did not induce this response, was acylated preferentially by phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Other in vitro studies, showing that PUFAs can be good substrates for LO-catalysed peroxidation even when they are esterified to phospholipids and cholesterol[26,27], have provided another potential route for the biosynthesis of HETE-containing phospholipids.

The present study was aimed at investigating further the metabolism of PUFAs in *H. vulgaris* and at establishing whether the LO products previously isolated from this species[20] could also be found esterfied to phospholipids, triglycerides and diacylglycerols.

Materials and methods

Hydra vulgaris was grown as previously described[18–20], and labelled with [^{14}C]-α-LA (NEN, 52 mCi/mmol, 0.5 µCi/ml, total incubation volume 2 ml) for 18 h. After five washes with 5 ml culture medium, the polyps (1000 specimens) were extracted three times with 10 ml chloroform/methanol (2:1 vol:vol). This method, due to the small volume of tissue to be extracted (<2 ml) and to the presence of some residual culture medium, which contains 1 mM $CaCl_2$, is generally considered to be sufficient for the extraction in good yields of all phospholipid classes, even the more polar ones such as mono- and di-phosphoinositides[28]. The organic phase was extracted three times with an equal volume of 0.05 M NH_4OH solution to get rid of free fatty acids. This latter step is necessary, since endogenous, radiolabelled ω-10-LO products (present as free acids)[20], may hide those produced from the hydrolysis and/or methanolysis of phospholipids (see below), but does not permit the use of a chloroform-methanol-HCl extraction (usually advised for the extraction of tri-phosphoinositides[28]) which, therefore, was not performed in the study. The organic phase was evaporated under vacuum, and the residue was loaded onto semipreparative t.l.c. silica-coated plates (Merck) which were developed with chloroform/methanol/water (64:25:2, vol:vol:vol). Under these conditions, standards of PE, PC, PI and phosphatidylserine (PS) (purchased from Sigma, U.K.) migrated with Rfs 0.7, 0.5, 0.3 and 0.1, respectively. More importantly, as shown by previous studies where hydroxy-fatty acid-containing phospholipids were isolated from mammalian cells[23,25], chloroform/methanol/water t.l.c. developing systems do not distinguish between 'hydroxylated-' and 'non-hydroxylated' phosphoglycerides, which comigrate at the same Rf. The four phospholipid classes were then scraped off the plates and eluted with chloroform/methanol (1:4 vol:vol). Total phospholipids or PC, PI, PE and PS were then submitted either to 1 h digestion at 37 °C with 10 units of PLA_2 (from bovine pancreas, purchased from Sigma), in a Tris-HCl 0.05 M buffer containing 1 mM $CaCl_2$, or reacted with anhydrous Na_2CO_3 in anhydrous methanol overnight at room temperature. PLA_2 incubates were extracted with chloroform/methanol (2:1 vol:vol) and the organic phases evaporated under vacuum before being submitted to reverse-phase HPLC (Spherisorb ODS-2 semipreparative column) using a slight modification of the elution conditions described previously[18–20], and consisting of a 40 min gradient from 55% to 65% acetonitrile in 0.1% trifluoroacetic acid (TFA)/water, followed by a 20 min wash with 100% acetonitrile in 0.1% TFA, flow rate 2 ml/min. Using these conditions, it is possible to separate with good resolution all the ω10-LO products of C_{18}, C_{20} and C_{22} PUFAs. The products of methanolysis were analysed using the same column eluted with a gradient over 1 h from 65% to 90% acetonitrile/0.1% TFA in 0.1% TFA/water (flow rate 2 ml/min). Standards of 9-HOTrE, 9-HPOTrE and 9-KOTrE, which are not commercially available, were prepared from incubation of homogenates of *H. vulgaris* with α-LA, carried out as described previously[18–20], followed by HPLC purification and [^{1}H]-NMR characterization. [^{1}H]-NMR spectra were identical to those previously reported for these three compounds[19,20]. HPLC fractions were finally submitted to scintillation counting, after addition of 10 ml scintillation liquid (Packard, U.K.). No quenching corrections were made.

Phospholipids, extracted from polyps pre-labelled for 18 hours (0.5 µCi/ml *Hydra* culture medium) with [^{3}H]-AA (NEN, 60 Ci/mol) or [^{14}C]-LA (NEN, 53 mCi/mmol), and washed 5 times with culture medium, were purified as described above, and submitted to either enzymatic hydrolysis (PLA_2, see above) or methanolysis, prior to analysis by HPLC, using the conditions described above. Commercially available 11-HETE, 11-HPETE, 9-HODE, 9-HPODE and 9-KODE (Biomol, U.K. and Cayman Chemicals, USA), as free acids or methyl esters, were used as standards.

Finally, micelles of either L-α-1-palmitoyl-2-linoleoyl-phosphatidyl-ethanolamine, L-α-1-stearoyl-2-arachi-

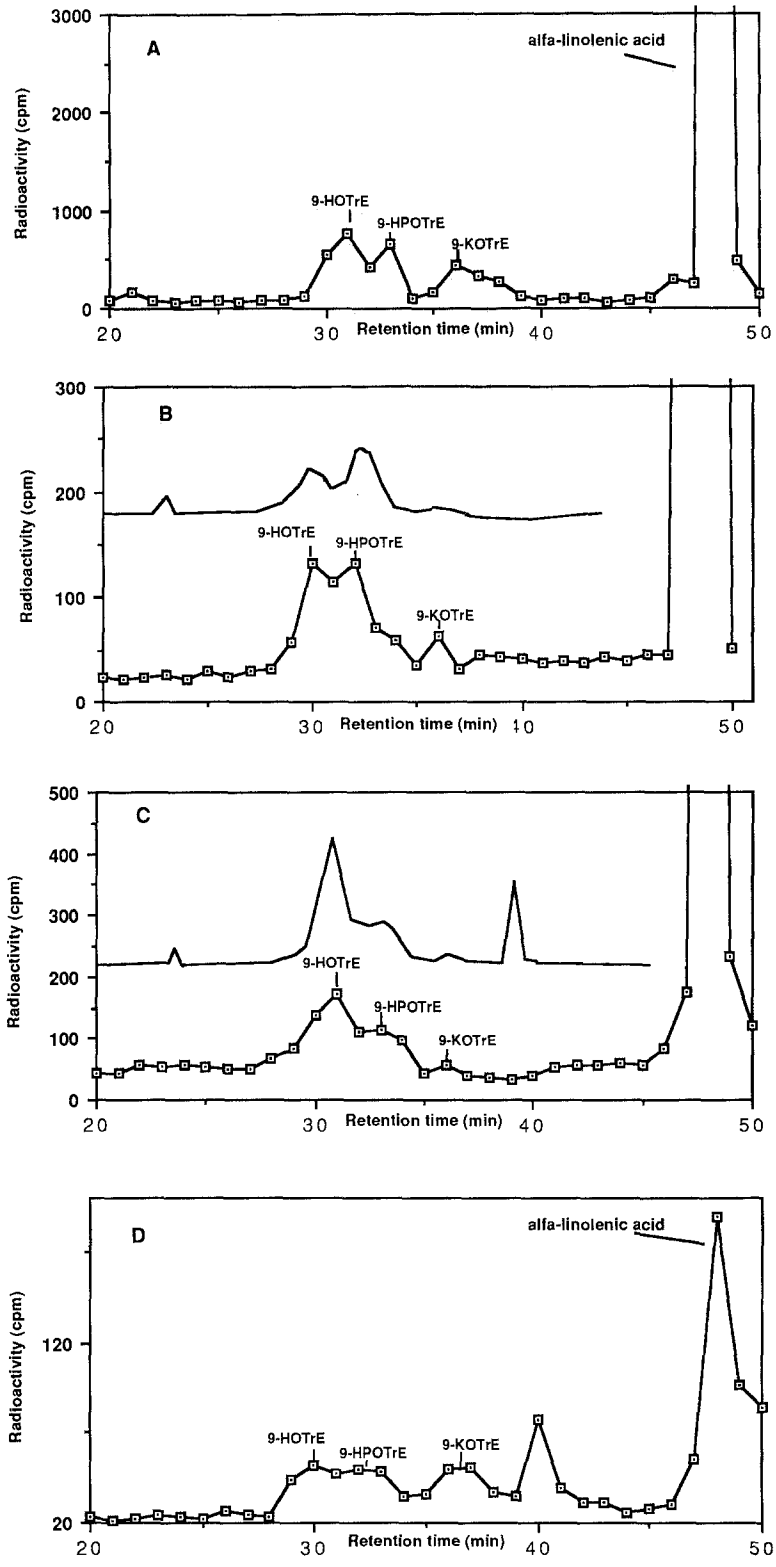

Figure 1. Typical radioactivity (cpm) profiles of HPLC analyses of samples from PLA_2 digestion of total phospholipids (*A*), phosphatidylethanolamine (*B*), phosphatidylcholine (*C*), and phosphatidylinositol (*D*) extracted from 1000 *H. vulgaris* polyps prelabelled by a 16 h incubation with [^{14}C]-α-LA (0.5 μCi/ml, 2 ml incubation volume). The retention times of 9-HOTrE, 9-HPOTrE and 9-KOTrE standards, prepared by incubating unlabelled α-LA with *H. vulgaris* homogenates[19], and co-injected with the samples, are shown. UV (234 nm) profiles are also shown in (*B*) and (*C*). These profiles were obtained from the analysis of non-radiolabelled samples which were not mixed with 9-HOTrE, 9-HPOTrE and 9-KOTrE standards prior to injection. The chromatograms are representative of three independent experiments.

donoyl-phosphatidyl-choline (Sigma, 1 mg/ml), or soybean phosphatidylinositol (Sigma, containing primarily palmitic and linoleic acid) and of L-α-1-palmitoyl-2-[^{14}C]-arachidonoyl-phosphatidyl-choline (NEN, 40–60 mCi/mmol, 1 μCi/ml) were prepared by sonication, added to homogenates of *H. vulgaris* (obtained by homogenization of polyps in 50 mM Tris-HCl buffer, pH = 7.4, and subsequent centrifugation at 7000 *g*, and containing approximately 1 mg/ml total proteins), and incubated for 1 h at 20 °C. Control incubations were

Table 1. Incorporation of radioactivity/100 specimens into α-linolenic acid metabolites (9-HOTrE plus 9-HPOTrE plus 9-KOTrE) produced from the enzymatic hydrolysis or methanolysis (*) of *H. vulgaris* neutral lipids.

Lipid	% incorporation	α-linolenic acid incorporation (cpm)
TAGs*	3.3 ± 1.8	2800 ± 1040
DAGs*	4.4 ± 1.9	1075 ± 650
PE	8.9 ± 2.2	4802 ± 2590
PC	5.4 ± 1.6	3215 ± 1990
PI	N.M.	283 ± 89
PS	N.D.	N.D.

Data are expressed as percentage of the amount/1000 specimens of [^{14}C]-α-linolenic acid incorporated into each fraction. This amount is also shown. Data are means ± s.d. of three separate experiments. TAGs = triacylglyc-erols; DAGs = diacylglycerols; PE = phosphatidylethanolamine; PC = phosphatidylcholine; PI = phosphatidylinositol; PS = phos-phatidylserine; N.M. = not measurable; N.D. = not detectable.

carried out using heat inactivated homogenates (negative control) or active homogenates (containing the ω10-LO activity described previously[18–20]) plus α-LA (0.5 mg/ml, positive control). The latter conditions were shown previously to be optimal for AA, α-LA and LA peroxidation by *H. vulgaris* (ω10-LO[18–20]). After the incubation, phospholipids were extracted with chloroform:methanol 2:1 (vol:vol), the organic phase was evaporated under vacuum, and the residue submitted to either enzymatic hydrolysis (PLA_2, see above) or methanolysis. The reaction products were then analysed by HPLC, carried out as described above.

Results and discussion

Extraction of lipids from 1000 polyps labelled with [^{14}C]-α-LA, followed by t.l.c. purification of phospholipids, incubation of the latter with porcine pancreatic PLA_2, and HPLC purification of the incubation mixture, yielded three radioactive components having the same retention times (under highly resolving chromatographic conditions[19,20]) as those of standards of 9-HOTrE, 9-HPOTrE and 9-KOTrE (fig. 1a). The percent incorporation of α-LA into each of the three matabolites is shown in table 1. HPLC analysis of fatty acid methyl esters obtained from the methanolysis of radiolabelled phospholipids yielded three radioactive peaks co-eluting with standards of 9-HOTrE, 9-HPOTrE and 9-KOTrE methyl esters (data not shown). Lower radioactivity was found in the corresponding compounds in HPLC analyses of the methanolysis products of *H. vulgaris* tri- and di-glycerides (table 1). These data strongly suggest the presence of 9-HOTrE, 9-HPOTrE and 9-KOTrE as acyl substituents on the 2-position of *H. vulgaris* phosphoglycerides. In order to assess the distribution of these metabolites among the four major classes of glycerophospholipids, total radiolabelled phospholipids from the hydrozoan were fractionated by t.l.c. as described in the Methods, and each phospholipid class separately submitted to PLA_2 digestion or methanolysis. The fatty acids (or fatty acid methyl esters) obtained were again purified by HPLC. As shown in figure 1(b–d), radioactivity peaks co-eluting with 9-HOTrE, 9-HPOTrE and 9-KOTrE standards were observed in PC and PE, but were hardly detectable in PI and absent in phosphatidylserine (PS, not shown). The percent radioactivity associated with α-LA metabolites compared to that associated with α-LA was similar for PC and PE (see table 1). In all cases, total incorporation of metabolites into each phospholipid class reflected the degree of α-LA incorporation, which is high in PC and PE, lower in PI and not detectable in PS (table 1). Similar results were obtained by HPLC analysis of the methanolysis products of the four phospholipid classes. In the case of PE and PC, the amount of 9-HOTrE-methyl ester released by quantitative methanolysis was sufficient to run a UV spectrum (not shown) which exhibited a maximum of absorbance at 235 nm, as expected from this type of compound.

In order to assess whether metabolites derived from the action of *H. vulgaris* ω10-LO on other PUFAs could also be found esterified to phospholipids, 1000 polyps were labelled with [^{3}H]-AA or [^{14}C]-LA and then extracted as before. Phospholipids were again submitted to either PLA_2 digestion or methanolysis, and the products analyses by HPLC in comparison with standards of ω10-LO-derived metabolites of AA and LA, i.e. 11-hydroxy- and 11-hydroperoxy-eicosa-5*Z*,8*Z*,12*E*,14*Z*-tetraenoic acid (11-HETE and 11-HPETE) and 9-hydroxy-, 9-hydroperoxy- and 9-keto-octadeca-10*E*, 12*Z*-dienoic acid (9-HODE, 9-HPODE and 9-KODE), as free acids or methyl esters. As shown in figure 2 (a, b), incorporation of radioactivity was found in the HPLC fractions corresponding to LA and, to a much smaller extent, AA derivatives.

Interestingly, a linear correlation was observed between the total radioactivity/1000 specimens incorporated into PUFA metabolite-containing phospholipids and the average amounts/1000 specimens of free α-LA, AA and LA (measured in previous studies[6,19,20]) (fig. 3), which, in turn, are proportional to the amounts of α-LA-, AA- and LA-derivatives found as free acids in *H. vulgaris* (ref. 20 and unpublished observations). Conversely, no correlation was seen between total radioactivity incorporated into PUFA metabolite-containing phospholipids and average amounts of PUFAs bound to phospholipids (fig. 3). These data seem to suggest that hydroperoxy-, hydroxy- and keto-fatty acid-containing phosphoglycerides are not derived from direct peroxidation of PUFA-containing phosphoglycerides, but, as in mammals, are rather the product of acylation of hydroperoxy-, hydroxy- and keto-fatty acids into the 2-position of phospholipids. However, since some LOs

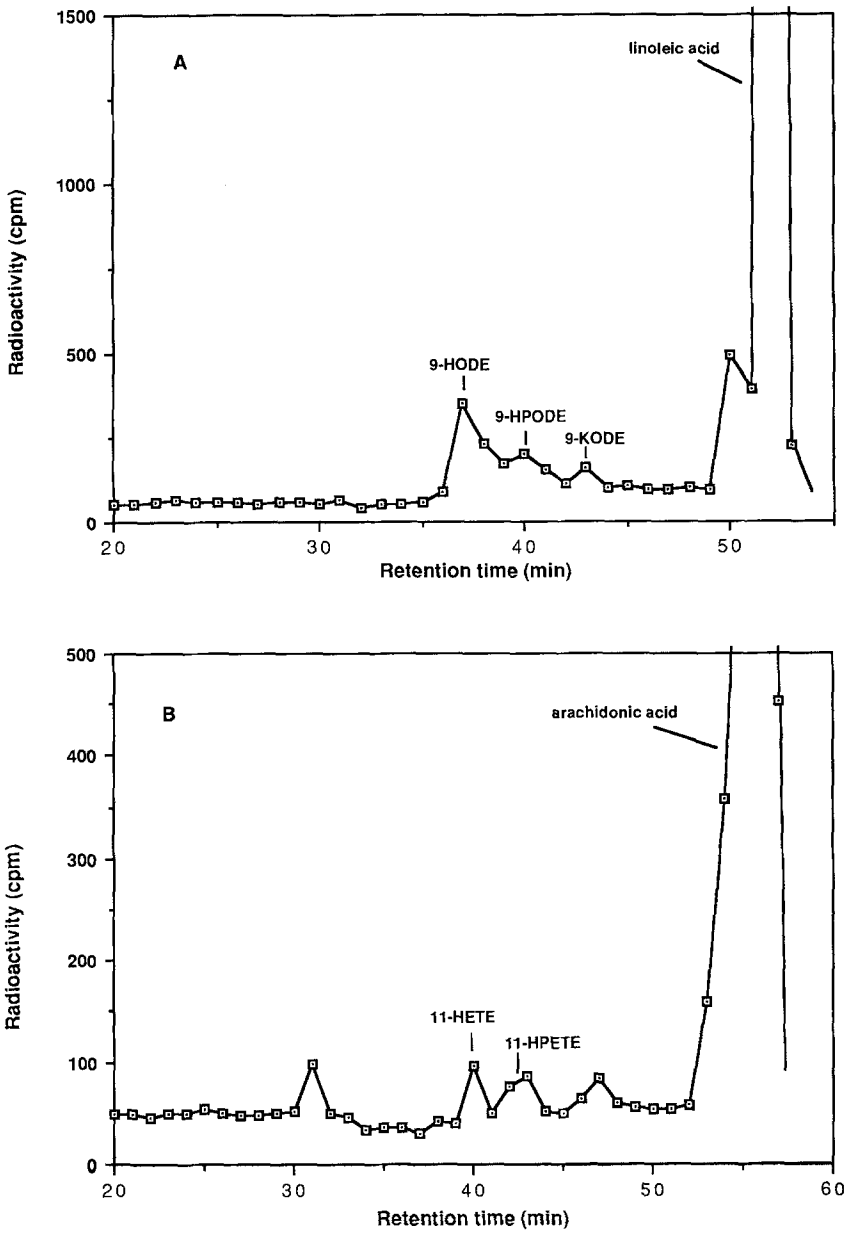

Figure 2. Typical radioactivity (cpm) profiles of HPLC analyses of samples from PLA_2 digestion of total phospholipids obtained from *H. vulgaris* polyps prelabelled by a 16 h incubation with (*A*) [^{14}C]-LA (0.5 µCi/ml, 2 ml incubation volume), or (*B*) [^{3}H]-AA (0.5 µCi/ml, 2 ml incubation volume). The retention times of 9-HODE, 9-HPODE, 9-KODE, 11-HETE and 11-HPETE authentic standards, which were co-injected with the samples, are shown. The chromatograms are representative of three independent experiments.

have recently been shown to recognize PUFAs when they are esterified to phospholipids and cholesterol[26,27], other experiments were designed in order to test this possibility for *H. vulgaris* ω10-LO definitively. In separate incubations, micelles of synthetic 2-[^{14}C]-arachidonoyl-PC, 2-linoleoyl-PE, 2-arachidonoyl-PC and natural PI (which contains high levels of LA esterified to its 2-position) were added to enzymatically active, ω10-LO-containing homogenates from *H. vulgaris*. After 1 h incubations, the reactions were stopped, and the phospholipids extracted and submitted to enzymatic hydrolysis or methanolysis followed by HPLC analysis, as described above. Incubates from positive controls, carried out with α-LA instead of phospholipids, were extracted and directly submitted to HPLC, and yielded high amounts of α-LA metabolites (not shown). In samples from incubations of homogenates with phosphoglycerides, however, no UV or radioactivity peak was found at the retention times of the PUFA-metabolite standards used, thus suggesting that phospholipid-bound PUFAs do not generate PUFA-metabolite-containing phosphoglycerides by spontaneous or ω10-LO-catalysed peroxidation. The possibility that enzymes different from the previously reported ω10-LO, and destroyed during the preparation of homogenates, might in vivo catalyse the peroxidation of PUFA-containing phospholipids cannot be ruled out by our data. Incubation of intact polyps with exogenous radiolabelled phosphoglycerides, which is likely to result in their incorporation into membranes and subsequent ex-

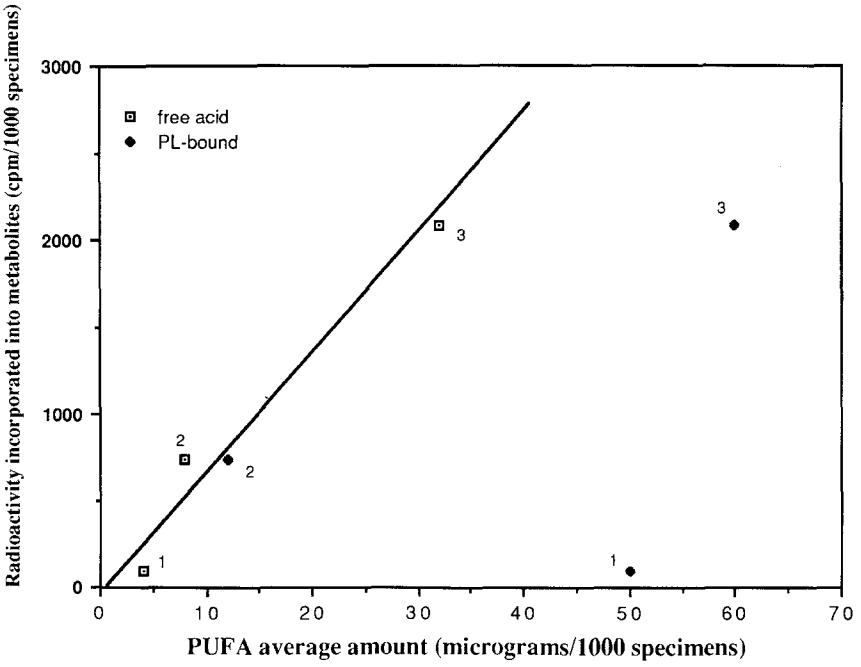

Figure 3. Correlation between the average amounts of endogenous free (□) and phospholipid-bound (◆) PUFAs in *H. vulgaris* (determined in previous studies[6,19–20]) and the radioactivity associated with the corresponding ω10-LO metabolites released upon methanolysis of *H. vulgaris* phospholipids. Numbers 1, 2 and 3 refer, respectively, to AA, LA and α-LA. Therefore, the amounts reported on the *x* axis are the average amounts/1000 specimens of free (□) and phospholipid-bound (◆) AA (1), LA (2) and α-LA (3); the radioactivity reported on the *y* axis is that contained in 11-HETE plus 11-HPETE (1), or 9-HODE plus 9-HPODE plus 9-KODE (2) or 9-HOTrE plus 9-HPOTrE plus 9-KOTrE (3), after HPLC fractionation of the samples from the methanolysis of total phospholipids extracted from 1000 polyps labelled respectively with [^{3}H]-AA, [^{14}C]-LA and [^{14}C]-α-LA.

change of the radiolabelled fatty acids with *Hydra* phosphoglycerides (thus preventing any conclusion being drawn on the possible oxidation of such lipids in vivo), was not attempted.

Finally, direct evidence for acylation of 9-HOTrE, 9-HPOTrE and 9-KOTrE into phospholipids was sought by attempting to incorporate into the latter metabolites radiolabelled 9-HOTrE obtained from a previous experiment where [^{14}C]-α-LA was incubated with ω10-LO-containing homogenate from *H. vulgaris* (radiolabelled 9-HOTrE, 9-HPOTrE and 9-KOTrE are not commercially available). Unfortunately, the very low specific radioactivity (1.2 mCi/mmol) of the compound prepared using this source prevented the detection of other than very little radioactivity in *H. vulgaris* phospholipids (64 ± 20 cpm/1000 specimens, after subtraction of background radioactivity, mean ± s.d., n = 3). However, since the 2-(9-HOTrE)-, 2-(9-HPOTrE)- and 2-(9-KOTrE)-phosphoglycerides detected can originate only from one of the two alternative pathways mentioned in this study (i.e. the 'acylation' or the 'direct peroxidation' pathway), and the 'direct peroxidation' route having found no experimental support from the data described above, it seems reasonable to suggest that the finding of α-LA metabolites esterified to PC, PE and, in trace amounts, PI, is due to ω10-LO-catalyzed peroxidation of α-LA followed by incorporation of peroxidation products into phospholipids. In any event, the present report represents the first example of the finding, in an invertebrate species, of LO products esterified to phospholipids. As in mammals, and, particularly, in polymorphonuclear neutrophils[24], the physiological significance of these chemically modified phospholipids may be that of a means for the 'storage' of potentially bioactive metabolites ready to be released upon stimulation of PLA_2. *H. vulgaris* contains high levels of a Ca^{2+}-activated membrane-bound PLA_2[9] whose activation by physiological stimuli may lead to the direct release of 9-HOTrE, 9-HPOTrE and 9-KOTrE as well as of their PUFA precursor, α-LA. Due to the preferential incorporation of these metabolites into PC and PE rather than PI, this 'remodeling' of phospholipids is unlikely to play a role in PI-mediated transmembrane signalling events (as was suggested for the specific acylation of 15-HETE to the 2-position of PI in bovine pulmonary arterial endothelial cells[23] and in rat cardiomyocytes[25]), but may rather affect the phospholipase D-mediated second messenger system, whose existence in hydroids, however, is yet to be shown. Unlike 15-HETE, and more like 5-HETE and 12-HETE in mammals[22–25], esterified 9-HOTrE and 9-KOTrE (and, possibly, 9-HODE) are found mainly in PC and PE, thus suggesting that in *Hydra* there might be acyl transferases catalysing the esterification (or *trans*-esterification) of the 2-position of phospholipids with different substrate selectivities. Since the degree of total incorpo-

ration of α-LA metabolites into phosphoglycerides reflects that of α-LA, one may speculate that the enzyme responsible for α-LA re-acylation into phospholipids also recognizes α-LA peroxidation products.

In conclusion, the present report has provided evidence for acylation of phospholipids by LO products in *Hydra*, thus extending to invertebrates a well-established concept in mammals, and confirming that hydrozoans can be taken as a useful and simple model for the study of the biosynthesis and metabolism of oxygenated fatty acids.

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