Two phosphatidylethanol classes separated by thin layer chromatography are produced by phospholipase D in rat brain hippocampal slices

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Abstract **Noradrenaline- and ionomycin-stimulated as well as basal phospholipase D activity from rat hippocampus produced, in the presence of ethanol, two different classes of [^{32}P]phosphatidylethanol (designated I and II), which were separated by thin layer chromatography. Endogenous labeling experiments using ^{3}H-fatty acids showed that two different classes of phosphatidylcholine, separated by two-dimensional TLC, one enriched with high incorporation of [^{3}H]arachidonic acid (B) and the other with [^{3}H]myristic acid (A), were the most likely sources for the two classes of phosphatidylethanol. Experiments where individual ^{32}P-phospholipids extracted from [^{32}P]Pi-labeled hippocampal slices were incubated with cabbage phospholipase D, in the presence of ethanol, showed that each class of [^{32}P]phosphatidylcholine, i.e. A and B, produced a different band of [^{32}P]phosphatidylethanol, with the same mobility in TLC as phosphatidylethanol II and I, respectively.**

Key words: Phospholipase D; Phosphatidylethanol; Rat hippocampus

1. Introduction

Phospholipase D (PLD), which was first described in plants and microorganisms, is known to be activated in mammalian tissues and cell lines. A variety of stimuli results in PLD stimulation. These include calcium mobilizing neurotransmitters and hormones, protein kinase C activators, and growth factors. There is growing evidence for a role of PLD in cell signaling [1,2]: the cleavage of the phosphodiester bond on the polar side of glycero-phospholipids catalyzed by PLD leads to the generation of phosphatidic acid (PtdOH) and the polar head group. PtdOH in addition to its own potential effects may act as a source of diacylglycerol (DAG) after being hydrolyzed by PtdOH phosphohydrolase. Phosphatidylcholine (PtdCho) has been considered as the main substrate for PLD [3–7]. However, several studies using exogenous substrates [8,9] or labeled endogenous phospholipids [10–14] have shown that other phospholipids or lysophospholipids [15] are also hydrolyzed by PLD in many types of intact cells or subcellular fractions. Substrate specificity has been related to distinct molecular forms of the enzyme localized in different subcellular fractions [16–18] or to different sensitivity to regulatory mechanisms [19,20]. A specific feature of PLD is the transphosphatidylation reaction, in which the enzyme transfers the phosphatidyl moiety of phospholipids to a short-chain primary alcohol and forms a metabolically stable phosphatidylalcohol, which is easily separated from the rest of naturally occurring phospholipids by thin layer chromatography (TLC). In the present study we have identified two different classes of phosphatidylethanol (PtdEtOH) generated after PLD stimulation in rat hippocampal slices. We suggest that two molecular classes of PtdCho may be the main substrates for PLD in rat hippocampus.

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Abbreviations: PLD, phospholipase D; PtdEtOH, phosphatidylethanol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdOH, phosphatidic acid; DAG, diacylglycerol; TLC, thin layer chromatography

2. Materials and methods

2.1. Materials

Noradrenaline, phospholipid standards and cabbage PLD were purchased from Sigma, ionomycin from Calbiochem, and [9,10-^{3}H(N)]myristic acid (30.7 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid (200 Ci/mmol), and [^{32}P]orthophosphoric acid from Amersham.

2.2. Preparation of slices and lipid labeling

Rat brain hippocampal slices (350×350 μm) were prepared as described previously [21]. The slices were incubated in 3 volumes of Krebs-Henseleit buffer without phosphate, containing 50 μCi/ml of [^{32}P]orthophosphoric acid alone or together with 30 μCi/ml [^{3}H]arachidonic acid or 30 μCi/ml [^{3}H]myristic acid for 2 h at 37°C, gassing the atmosphere with O_2/CO_2 (95:5 v/v) every 30 min. After labeling, the slices were washed with buffer and settled under gravity.

2.3. PLD stimulation and measurement of PtdEtOH

Twenty-microliter samples of labeled slices (0.8 mg protein, approximately) were incubated with agonists and ethanol (200 mM final concentration) as previously described [21]. To separate [^{32}P,^{3}H]PtdEtOH from the other ^{32}P,^{3}H-lipids in lipid extracts, these were resuspended in 15 μl chloroform/methanol (4:1 v/v) and spotted onto silica gel-60 TLC plates (Merck) that were developed with chloroform/methanol/acetic acid (65:15:2 v/v). The area corresponding to [^{32}P,^{3}H]PtdEtOH was identified with authentic standards, and the two bands were visualized after autoradiography, scraped and counted for radioactivity. ^{32}P,^{3}H-Phospholipids were separated by two dimensional TLC, using chloroform/methanol/ammonia (65:35:10 v/v) in the first dimension and chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1 v/v) in the second dimension. Iodine-labeled and/or autoradiographically visualized spots were identified with authentic phospholipid standards.

2.4. Preparation of [^{32}P]Pi-labeled phospholipids and transphosphatidylation by cabbage PLD

^{32}P-Phospholipids, separated by two-dimensional TLC as described above, were scraped into microfuge tubes and extracted from silica gel by adding 0.6 ml of chloroform/methanol (1:1 v/v). After a short sonication (30 s) and a 5-min centrifugation at maximum speed, 0.4 ml of supernatants were transferred to assay tubes. The silica gel was washed with 0.5 ml of the same mixture and the supernatants were pooled. The solvent was evaporated under vacuum and each of the individual ^{32}P-phospholipids was incubated with cabbage PLD as described in reference [22]. In brief, 10 units of PLD and ^{32}P-phos-

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pholipid from 40 μl of labeled slices (1.6 mg protein, approximately) were incubated in a final volume of 1 ml for 2 h at 37°C in 0.1 M acetate buffer, pH 5.6, containing 140 mM $CaCl_2$ and 1 M ethanol. Reactions were stopped by adding 1.2 ml chloroform/methanol (1:2 v/v) and the [^{32}P]PtdEtOH produced was separated, identified and visualized autoradiographically as described above.

3. Results

Fig. 1 shows the autoradiography obtained after TLC separation of [^{32}P]PtdEtOH produced upon PLD stimulation in [^{32}P]Pi-prelabeled slices from rat hippocampus. Under each condition (basal, 30 μM noradrenaline, and 10 μM ionomycin), two different bands appeared in the area corresponding to PtdEtOH (indicated as I and II, Rf = 0.40 and Rf = 0.35, respectively). Both of them correspond to [^{32}P]PtdEtOH as they were only formed in the presence of ethanol. These two bands also appeared after stimulation with other agents known to enhance PLD activity, like endothelin-1, histamine, trans-(1*S*,3*R*)-aminocyclopentyl-1,3-dicarboxylic acid or phorbol myristate acetate; when other primary alcohols, such as propanol or butanol, were used as acceptors of the phosphatidyl moiety of the PLD-cleaved phospholipids, two phosphatidylalcohol bands were also formed; furthermore, similar results were obtained using cortical instead of hippocampal slices (not shown).

Fig. 2 shows the autoradiography of a two-dimensional TLC separation of hippocampal ^{32}P-phospholipids, where it is worth noting that two spots (designated A and B) appeared in the area corresponding to PtdCho, and their identity was confirmed after spraying the plate with the choline-specific Dragendorff stain [23]. The different mobility of two [^{32}P]PtdEtOH bands in TLC (Fig. 1) could reflect a different fatty acid composition, and therefore we conducted double labeling experiments in order to compare the labeled fatty acid incorporation to each PtdEtOH to that to the major phospholipid classes separated as shown in Fig. 2. After labeling the slices with [^{32}P]Pi and either [^{3}H]myristic acid or [^{3}H]arachidonic acid, the distribution, on a percentage basis, of each radioactive marker among the two PtdEtOH bands as

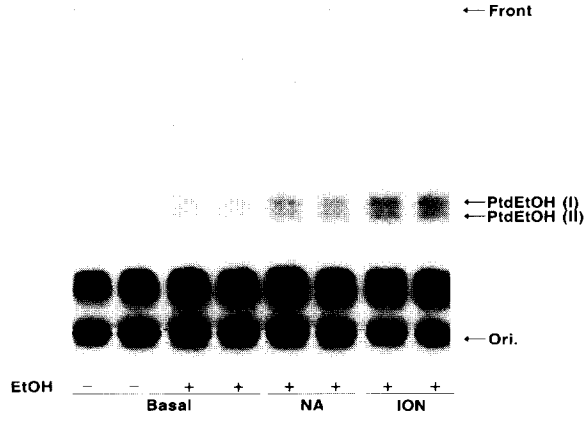

Fig. 1. Autoradiography of [^{32}P]PtdEtOH separated from other ^{32}P-phospholipids by TLC after PLD stimulation. 20 μl of [^{32}P]Pi-labeled hippocampal slices was incubated 30 min in the absence or the presence of 200 mM ethanol with no further addition (basal), 30 μM noradrenaline (NA), or 10 μM ionomycin (ION), and lipid extracts were processed as described in Section 2. The autoradiography shown represents an example of eight experiments in quadruplicate.

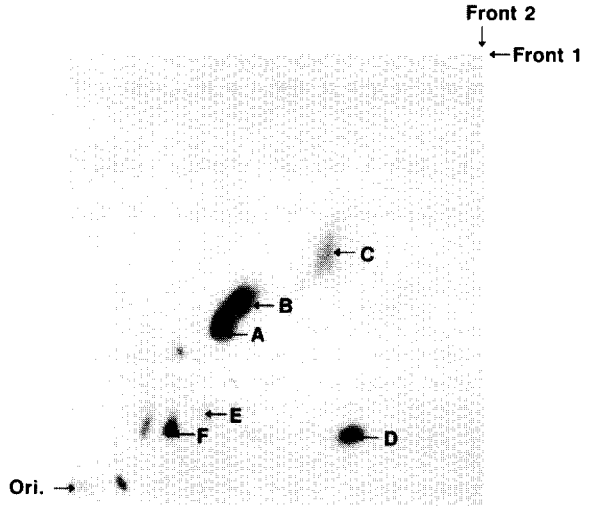

Fig. 2. Autoradiography of ^{32}P-lipids separated by two-dimensional TLC. Phospholipids from 20-μl samples of [^{32}P]Pi-labeled slices were separated as described in Section 2. The major phospholipid classes were identified with authentic phospholipid standards and the spots corresponding to PtdCho were checked with the choline-specific Dragendorff staining (A and B, PtdCho; C, PtdEtn; D, PtdOH; E, PtdSer; F, PtdIns). The autoradiography shown represents an example of eight experiments in quadruplicate.

compared to that in phospholipids could indicate the most likely substrates for PLD in rat brain. Fig. 3 shows the results of such experiments, expressed as percent distribution among phospholipids (upper panel) and the two phosphatidylethanol bands (lower panel).

PtdCho (A) was enriched with [^{3}H]myristic acid and had a negligible content of [^{3}H]arachidonic acid. In contrast, the percent incorporation of [^{3}H]arachidonic acid in PtdCho (B) was two-fold that for [^{3}H]myristic acid. [^{32}P]Pi label was similar in both molecular classes of PtdCho. Phosphatidylethanolamine (PtdEtn) had an equal percentage for the three markers, phosphatidylinositol (PtdIns) had a high incorporation of [^{3}H]arachidonic acid, which was two times higher than the [^{32}P]Pi incorporation, and less than 5% of the three labeled precursors was found in phosphatidylserine (PtdSer). On the other hand, the PtdEtOH with higher mobility (I) was enriched in [^{3}H]arachidonic acid, and the slower PtdEtOH (II) showed a relatively higher content of [^{3}H]myristic acid. Furthermore, expressing the percent content of a given labeled precursor in PtdEtOH relative to that of total lipids we observed the following results: PLD stimulation, calculated considering [^{32}P]Pi labeling, paralleled [^{3}H]arachidonic acid labeling in PtdEtOH (I) and [^{3}H]myristic acid labeling in PtdEtOH (II).

These results suggest that both [^{3}H]myristic acid-enriched (A) and [^{3}H]arachidonic acid-enriched (B) PtdCho may be substrates for PLD in rat hippocampus, the first one being the most likely source for the slower PtdEtOH (II) and the second one for the faster PtdEtOH (I). Although PtdIns, PtdEtn and PtdSer do not appear to be the main substrates for PLD they cannot be excluded as a minor source of PtdEtOH production.

Another approach to further investigate the involvement of each phospholipid class in the production of the two different PtdEtOHs was as follows: phospholipids from [^{32}P]Pi labeled slices were separated by TLC and extracted from silica gel as

described in Section 2 prior to being incubated individually with PLD from cabbage. After transphosphatidylation in the presence of 1 M ethanol, PtdEtOH was separated by TLC. The autoradiography in Fig. 4 (upper panel) shows that the two different [^{32}P]PtdChos, when cleaved separately by PLD in the presence of ethanol, produced a single band of [^{32}P]PtdEtOH: [^{32}P]PtdCho (A) yielded the slower [^{32}P]PtdEtOH (II) while [^{32}P]PtdCho (B) produced the faster [^{32}P]PtdEtOH (I). When both classes of [^{32}P]PtdCho were simultaneously presented as substrates to cabbage PLD, two bands of [^{32}P]PtdEtOH were observed. Furthermore, in the absence of ethanol during the PLD reaction, the corresponding [^{32}P]PtdOH for each [^{32}P]PtdCho also showed distinct mobility. Finally, incubation of [^{32}P]PtdEtn and [^{32}P]PtdIns with cabbage PLD elicited a very slight production of [^{32}P]PtdEtOH (I) (hardly observed in Fig. 4, lower panel).

4. Discussion

Since it was first described in 1967 [24], the transphosphatidylation reaction has been used as an unequivocal index of PLD activity. The phosphatidylalcohols generated, which do not occur in nature, are separated from the rest of cellular phospholipids by thin layer chromatography onto silica gel plates. Several solvent systems have been developed to achieve that separation with good resolution [25]. However, among the solvent systems used, only with the mixture chloroform/methanol/acetic acid (65:15:2 v/v), two PtdEtOH bands were autoradiographically visualized. By contrast, using the organic phase of isooctane/ethylacetate/acetic acid/water (110:50:20:100 v/v), PtdOH and PtdEtOH were separated from each other and from the rest of phospholipids, but only one band appeared that was identified as PtdEtOH (not shown).

Our results indicate that two molecular classes of PtdCho are the most likely sources for the two PtdEtOHs generated by PLD in rat hippocampus. The two PtdChos were separated by TLC, and they were distinguished by their different relative incorporation of [^{3}H]myristic acid and [^{3}H]arachidonic acid, in good accordance with the content of these labeled fatty acids in the two PtdEtOHs. Although the substrate specificity of rat hippocampus and cabbage PLD may not be the same, results in Fig. 4 show that PtdCho (A) and (B) are cleaved by a phospholipase of the D type, and the products formed ex-

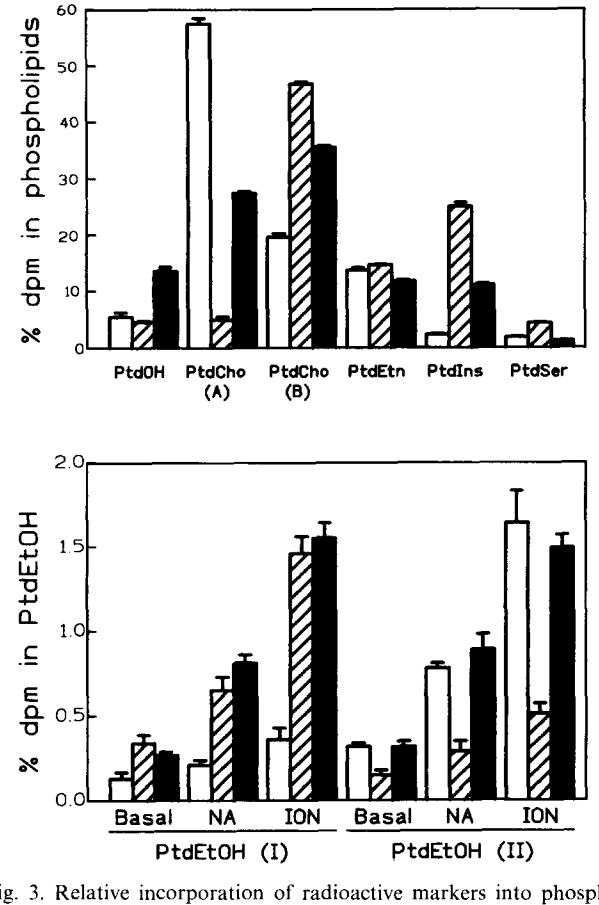

Fig. 3. Relative incorporation of radioactive markers into phospholipids and PtdEtOH. Slices were labeled with [^{32}P]Pi and either [^{3}H]myristic acid or [^{3}H]arachidonic acid. The content of [^{3}H]myristic acid (open bars), [^{3}H]arachidonic acid (hatched bars), and [^{32}P]Pi (filled bars) in each individual phospholipid was calculated as a percentage of the total radioactivity in the major phospholipids (PtdOH, PtdCho, PtdEtn, PtdIns, and PtdSer). Top: ^{32}P,^{3}H-lipids from 20-μl slices were separated by two-dimensional TLC, scrapped and counted for radioactivity. Bottom: 20-μl slices were stimulated with 30 μM noradrenaline (NA) or 10 μM ionomycin (ION) in the presence of 200 mM ethanol, then [^{32}P,^{3}H]PtdEtOH was separated by TLC. Results are means ± S.E.M. of four experiments in quadruplicate.

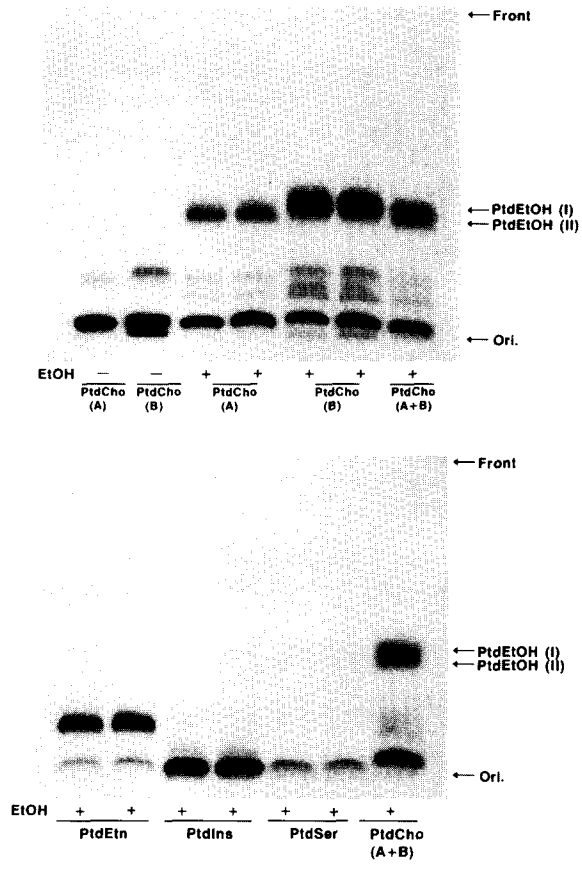

Fig. 4. Autoradiography of [^{32}P]PtdEtOH produced by cabbage PLD acting on individual ^{32}P-phospholipid classes from rat hippocampus. Individual ^{32}P-phospholipid classes obtained from 40 μl of [^{32}P]Pi-labeled slices were incubated with cabbage PLD in the absence or the presence of 1 M ethanol for 1 h. The [^{32}P]PtdEtOH generated was separated as described in Section 2. This experiment was repeated three times with the same results.

hibit in TLC the same mobility as PtdEtOH (II) and PtdEtOH (I), respectively.

We do not know either the molecular species that may belong to PtdCho A or B class, or whether [^{3}H]myristic acid or [^{3}H]arachidonic acid incorporation reflects the real content of both fatty acids in the two PtdChos. However, we have found that [^{3}H]palmitic acid incorporation to both PtdEtOHs was similar to that of [^{3}H]myristic acid (not shown), suggesting that the two PtdEtOH species differ in their content and/or labeling of arachidonate and saturated fatty acids.

Finally, [^{32}P]PtdEtOH (% of ^{32}P-lipids), as a quantitative measure of PtdEtOH production, indicate that two different types of PLD stimulation, α_1-adrenoceptor-mediated and direct calcium gating by ionomycin, result in similar proportions of both classes of PtdEtOH. In contrast, the ^{3}H-fatty acid content of each PtdEtOH differs markedly and may not adequately reflect the bulk production of PtdEtOH.

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