The substrate specificity of brain microsomal phospholipase D

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Neurotransmitters activate a phospholipase D that is thought to specifically hydrolyse phosphatidylcholine. This enzyme has a unique property known as transphosphatidylation: in the presence of an appropriate nucleophilic receptor such as an alcohol, phospholipase D will catalyse the production of phosphatidylalcohol. We have studied phospholipase D using an *in vitro* assay that uses [^{3}H]butanol of high specific radioactivity (15 Ci/mmol) as an acceptor. In the presence of [^{3}H]butanol and phosphatidylcholine, a microsomal membrane fraction from rat brain catalysed the production of phosphatidyl[^{3}H]butanol. Phospholipase D activity was dependent upon the presence of a detergent; the optimal sodium oleate concentration was between 4 and 6 mM. The R_F of the phosphatidyl[^{3}H]butanol on t.l.c. was identical to the R_F of the phosphatidylbutanol formed when [^{3}H]phosphatidylcholine was incubated with 100 mM butanol. These data confirm the identity of phosphatidyl[^{3}H]butanol. One important advantage of this assay is that the substrate does not need to be labelled. We have used this advantage to examine the substrate specificity of phospholipase D. Microsomal phospholipase D appears to hydrolyse phosphatidylcholine most efficiently. There is a relatively small but significant activity against phosphatidylethanolamine and phosphatidylserine, and there is no significant activity against phosphatidylinositol. As the headgroup becomes more like choline, the phospholipid becomes a better substrate for phospholipase D. The addition of one methyl group leads to a large increase in activity. Fatty acid composition does not play a role in determining the substrate specificity. This assay should be useful in furthering our understanding of this important enzyme.

INTRODUCTION

The effect of neurotransmitters on diacylglycerol production was thought to be the result of the activation of a phospholipase C that hydrolyses inositol-containing phospholipids. However, recent research indicates that the pathways of neurotransmitter-stimulated phospholipid metabolism are more complex and that diacylglycerol can be derived from phosphatidylcholine in addition to phosphatidylinositol (Hughes et al., 1984; Bocckino et al., 1985; Ragab-Thomas et al., 1987; Rosoff et al., 1988; Pessin and Raben, 1989; Horwitz, 1990). The hydrolysis of phosphatidylcholine can be mediated by two enzymes, phospholipase C and/or phospholipase D. The action of phospholipase C would result in the formation of diacylglycerol directly (Besterman et al., 1986). The action of phospholipase D would result in the formation of phosphatidic acid and choline (Witter and Kanfer, 1985); the hydrolysis of phosphatidic acid by phosphatases would then lead to the subsequent production of diacylglycerol. An early increase in phosphatidic acid and a delayed increase in diacylglycerol production are consistent with the early activation of phospholipase D (Bocckino et al., 1987; Horwitz, 1991).

Phosphatidic acid and choline are labile intermediates that can be formed by several different pathways, and as such are not the best indicators of phospholipase D activity. Phospholipase D has the unique property of catalysing the transfer of phosphatidyl groups to various acceptors. In the presence of an alcohol, this transphosphatidylation produces phosphatidylalcohol (Kobayashi and Kanfer, 1987). The production of phosphatidylalcohol is considered to be a good indication of phospholipase D activity. Investigations from many laboratories, including our own, indicate that neurotransmitters and hormones can cause a rapid increase in phosphatidylalcohol, reflecting an activation of phospholipase D (Exton, 1990; Billah and Anthes, 1990; Shukla and Halenda, 1991).

In PC12 cells, as well as in other neuronal tissues, the fatty acid composition of phosphatidylcholine differs significantly from that of the other three major phospholipids (Traynor et al., 1982). Previous results from this laboratory indicate that the fatty acid composition of bradykinin-stimulated phosphatidylethanol closely resembles the fatty acid composition of phosphatidylcholine (Horwitz, 1991). These data suggest that phospholipase D in the intact cell specifically hydrolyses phosphatidylcholine. These results have been confirmed by fast-atom-bombardment mass spectrometry analysis of phospholipid species (Holbrook et al., 1992). *In vitro* assays also suggest that membrane-bound phospholipase D from neuronal tissues is specific for phosphatidylcholine (Kobayashi and Kanfer, 1987; Wang et al., 1991; Mohn et al., 1992). Apparently, membrane-bound phospholipase D has an inherent specificity for phosphatidylcholine. This characteristic could account for the specificity of phospholipase D in intact cells.

In order to investigate the issue of substrate specificity in more detail, we have modified an assay for phospholipase D, first developed by Randall et al. (1990), that utilizes [^{3}H]butanol as an acceptor. One advantage of using [^{3}H]butanol of high specific radioactivity is that the substrate does not need to be labelled. Therefore it is possible to measure the activity of phospholipase D against different substrates that are commercially available but not yet available in radiolabelled form. An additional advantage is that we can measure the activity of phospholipase D against substrates at maximal concentration in the absence of high concentrations of ethanol. We have used this essay to measure the substrate specificity of phospholipase D from microsomal membrane preparations obtained from whole rat brain.

EXPERIMENTAL

Isolation of microsomal membranes

Crude microsomal membranes were isolated from whole rat brain. Brains were homogenized in 10 vol. of cold 0.32 M sucrose

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containing 1 mM EGTA. The crude homogenate was centrifuged for 10 min at 1000 *g*. The supernatant was then centrifuged for 30 min at 20000 *g*. Finally, the microsomal membranes were pelleted by centrifugation at 100000 *g* for 60 min. The pellet was then washed with 0.32 M sucrose. The membranes were resuspended at a concentration of 2 mg of protein/ml and stored at −80 °C until use.

Assay of phospholipase D activity

The assay conditions were adapted from the method of Kobayashi and Kanfer (1987). The phospholipid substrate obtained commercially was dried down under a stream of nitrogen and then resuspended in 36 mM sodium oleate in 5 mM β-dimethylglutaric acid, pH 6.5. This suspension was allowed to sit for 60 min at room temperature. Vesicle formation was induced by sonicating in a small bath sonicator at 20 s intervals for 10 min. At alternating intervals the tube was put on ice. Unless indicated otherwise, the assay conditions were: 2 mM phospholipid, 50 mM β-dimethylglutaric acid, pH 6.5, 10 mM EDTA, 6 mM sodium oleate, 5 μCi of [^{3}H]butanol or [^{3}H]ethanol (15 Ci/mmol; American Radiolabeled Chemical) and 100–200 μg of crude membrane protein in a final volume of 120 μl. Blanks contained 0.32 M sucrose rather than membranes. Incubations were carried out for 60 min at 30 °C. The reactions were stopped by adding 3 ml of ice-cold chloroform/methanol (1:2, v/v) and 0.7 ml of 1% perchloric acid. The mixture was vortexed for 1 min. The phases were separated by the addition of 1 ml of 1% perchloric acid and 1 ml of chloroform. After vortexing, the tubes were centrifuged and the upper phase was aspirated. The lower phase was then washed twice with 2 ml of 1% perchloric acid. An aliquot of 500 μl was removed and dried down for t.l.c.

In one experiment the substrate was L-α-dipalmitoyl-[2-*palmitoyl*-9,10-^{3}H(n)]phosphatidylcholine (specific radioactivity 2 mCi/mmol) prepared in the following way. Egg yolk phosphatidylcholine and [^{3}H]phosphatidylcholine were dried together in a glass tube under a stream of nitrogen and resuspended in 36 mM sodium oleate. Unlabelled butanol at a concentration of 100 mM was then added as an acceptor.

T.l.c. was routinely carried out according to Liscovitch (1989). Whatman LK6 plates were dipped in 1.3% potassium oxalate made up in methanol/water (2:3, v/v) and allowed to dry at room temperature. Just before use, the plates were activated by heating at 115 °C for 1 h. The mobile phase was the upper layer of a mixture of ethylacetate/iso-octane/acetic acid/water (13:2:3:10, by vol.). The t.l.c. scrapings were counted for radioactivity by scintillation spectrometry.

The commercially obtained phospholipids were separated by a two-step single-dimension chromatography system on h.p.l.c. plates (Takamura et al., 1987). They were then visualized by charring with a $CuSO_4$ solution (Banerjee et al., 1990). All the phospholipids were found to be pure.

Materials

Male Sprague–Dawley rats were obtained from ACE Animals (Boyertown, PA, U.S.A.) at 150–200 g body weight. [^{3}H]Butanol and ethanol at 15 Ci/mmol were obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). L-α-Dipalmitoyl-[2-*palmitoyl*-9,10-^{3}H(n)]phosphatidylcholine was from New England Nuclear (Boston, MA, U.S.A.). Phospholipids, sodium oleate and dimethylglutaric acid were obtained from Sigma (St. Louis, MO, U.S.A.). Phosphatidylethanol was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.).

RESULTS AND DISCUSSION

In order to verify the identity of phosphatidylbutanol, microsomal membranes were incubated in the presence of either [^{3}H]dipalmitoyl-phosphatidylcholine plus unlabelled butanol at a concentration of 100 mM or egg yolk phosphatidylcholine plus [^{3}H]butanol. Phosphatidylbutanol had the same R_F, 0.5, whether it was labelled in the fatty acid or in the butanol moiety (results not shown). Phosphatidylethanol obtained commercially has an R_F of approx. 0.38. These data confirm the identity of this compound as phosphatidylbutanol. In all future experiments this area of the thin-layer chromatogram was scraped.

Figure 1 shows the detergent-dependence of phospholipase D activity using phosphatidylcholine as a substrate. With 100 mg of protein per assay the optimal concentration of sodium oleate was 4–6 mM. The amount of detergent required is dependent on the amount of protein and the substrate concentration (Kobayashi and Kanfer, 1987). This detergent-dependence was apparent whether [^{3}H]butanol or [^{3}H]ethanol was used as substrate, although it was clear that [^{3}H]ethanol was not as efficient an acceptor. The specific activity of phospholipase C varied depending on the experiment and individual microsomal preparation. In this experiment the specific activity using [^{3}H]butanol as an acceptor was 6 pmol/h per mg and using [^{3}H]ethanol it was 1 pmol/h per mg. These values are much lower than those reported by Kobayashi and Kanfer (1987). This is probably due to the fact that the actual concentration of unlabelled alcohol in the assay was low, of the order of 2.8 μM for butanol and 1.9 μM for ethanol.

It has been suggested that longer-chain alcohols inhibit phospholipase D activity (Chalifa et al., 1990). From these data it is apparent that, at low concentrations, butanol is a much better acceptor than ethanol. The higher concentrations used by these authors, 200 mM, probably disrupted membrane structure. The difference in activity between [^{3}H]butanol and [^{3}H]ethanol is not due to an inaccurate estimation of specific activity, because we have obtained qualitatively similar results in another phospholipase D assay using unlabelled alcohols (results not shown). The

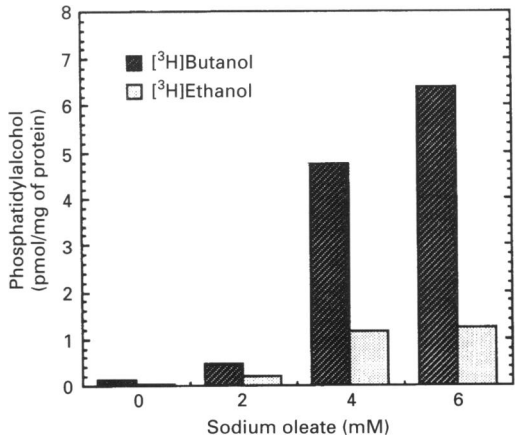

Figure 1 Detergent-dependence of phospholipase D activity in microsomal membranes

Phosphatidylcholine (egg yolk) was resuspended in different concentrations of sodium oleate by sonication; phospholipase D was assayed as described in the Experimental section. Each assay contained 5 μCi of either [^{3}H]butanol or [^{3}H]ethanol and phospholipid at a concentration of 2 mM. Results are means of duplicate determinations. The results are representative of at least two experiments.

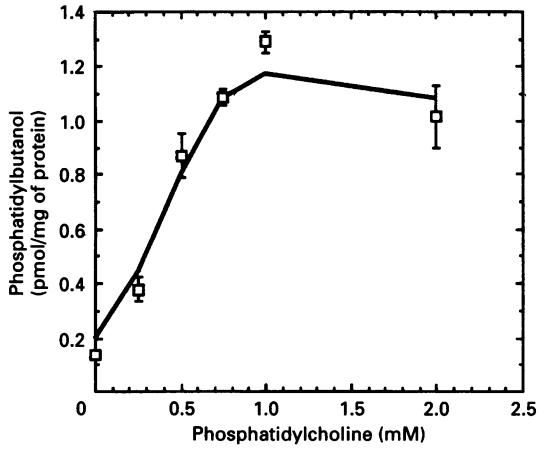

Figure 2 Concentration-dependence of phospholipase D activity in microsomal membranes

Different amounts of phosphatidylcholine (from egg yolk) were dried down under a stream of nitrogen and then resuspended by sonication in different concentrations of sodium oleate, such that the final detergent/phospholipid (mol/mol) ratio was 3; phospholipase D was assayed as described in the Experimental section. Each assay contained 5 μCi of [^{3}H]butanol. Results are means ± S.E.M. of triplicate determinations. The results are representative of at least two similar experiments.

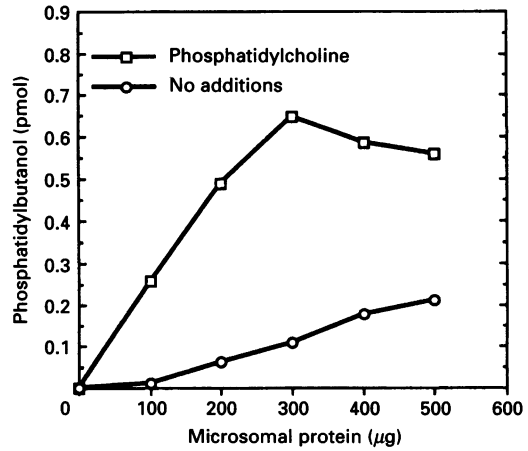

Figure 3 Protein-dependence of phospholipase D activity in microsomal membranes

Phosphatidylcholine from bovine brain was resuspended in sodium oleate by sonication such that the final concentration was 6 mM; the final concentration of phosphatidylcholine was 2.4 mM. Samples with no additional phospholipid still contained sodium oleate. Phospholipase D was assayed as described in the Experimental section. Each assay contained 5 μCi of [^{3}H]butanol and microsomal protein (100–500 μg). Each point represents the mean of duplicate determinations.

enhanced ability of longer-chain alcohols to act as acceptors may be due to their increased lipid solubility.

The concentration dependence of phospholipase D activity using egg yolk phosphatidylcholine as a substrate is shown in Figure 2. The detergent concentration was altered so that the detergent/phospholipid ratio was maintained at 3. The approximate EC_{50} for added phosphatidylcholine was 0.4 mM. Maximal levels of activity were obtained at a concentration of 1 mM. There was some phosphatidylbutanol production in the absence of added phosphatidylcholine. This suggested that endogenous phospholipids may be acting as substrates for phospholipase D. We examined this issue further by measuring phospholipase D activity as a function of protein concentration (Figure 3). In the absence of exogenous phosphatidylcholine, phosphatidylbutanol production increased as a function of protein concentration. This is consistent with the idea that endogenous phospholipids are able to be hydrolysed. In the presence of phosphatidylcholine, phosphatidylbutanol production was linear from 100 to 300 μg of protein. At higher protein concentrations there was a decrease in specific activity. This may be due to an alteration in the detergent/protein ratio or to competition by endogenous phospholipids that are not optimal substrates for the phospholipase D.

Table 1 Substrate specificity of phospholipase D in microsomal membranes

All phospholipids were derived from bovine brain, except for phosphatidylinositol which was derived from bovine liver. The final concentration of all phospholipids was 2 mM. Phospholipids were dried down and resuspended in sodium oleate by sonication such that the final concentration was 6 mM; phospholipase D was assayed as described in the Experimental section. Each assay contained 5 μCi of either [^{3}H]butanol or [^{3}H]ethanol and 200 μg of microsomal protein. Results are means ± S.E.M. of triplicate determinations. The results are representative of at least two similar experiments. *Significant difference from the no phospholipid control at the $P < 0.05$ level.

	Phospholipase D activity (pmol/mg of protein)	
	[^{3}H]Butanol	[^{3}H]Ethanol
No phospholipid	0.18 ± 0.02	0.050 ± 0.004
Phosphatidylinositol	0.26 ± 0.08	0.046 ± 0.003
Phosphatidylserine	0.34 ± 0.02*	0.074 ± 0.002*
Phosphatidylethanolamine	0.33 ± 0.01*	0.092 ± 0.012*
Phosphatidylcholine	1.32 ± 0.05*	0.329 ± 0.027*

This assay was then used to examine the activity of phospholipase D against unlabelled phospholipids obtained commercially. Table 1 shows the substrate specificity of microsomal phospholipase D. All of the phospholipids were from brain, except for phosphatidylinositol which was extracted from liver. The final concentration of all phospholipids was 2 mM. Phospholipase D hydrolysed phosphatidylcholine most efficiently. There was a relatively small but significant activity against phosphatidylethanolamine and phosphatidylserine. In this experiment there was no significant product formation when phosphatidylinositol was used as a substrate. However, in other experiments a small but significant activity against phosphatidylinositol was apparent. This substrate specificity profile was the same whether [^{3}H]ethanol or [^{3}H]butanol was used as an acceptor; however, as before, [^{3}H]butanol appeared to be more efficient. These results qualitatively agree with those of Kobayashi and Kanfer (1987). In their study, the activity against other phospholipids was less than 8% of that against phosphatidylcholine. In the present study it was 13–15%. Thus microsomal phospholipase D prepared from whole rat brain hydrolyses phosphatidylcholine preferentially but not exclusively.

As stated in the Experimental section, the phosphatidylethanolamine and phosphatidylserine used were pure and not contaminated with phosphatidylcholine. In addition, incubation of these phospholipids with the microsomal membrane extract under conditions identical to those of the assay did not cause their conversion to phosphatidylcholine (results not shown). Thus the residual activity against phosphatidylethanolamine and

Table 2 Specificity of microsomal phospholipase D for the choline headgroup

Dipalmitoyl analogues of the listed phospholipids were dried down under a stream of nitrogen and resuspended in sodium oleate, such that the final concentration was 6 mM. Each assay contained 5 μCi of [^{3}H]butanol and phospholipid at a concentration of 2 mM. Results are means ± S.E.M. of triplicate determinations. The results are representative of at least two similar experiments. *Significant difference from the no phospholipid control at the $P < 0.05$ level.

	Phosphatidyl-[^{3}H]butanol (pmol/mg of protein)
No phospholipid	0.27 ± 0.08
Phosphatidylethanolamine	0.59 ± 0.03*
Phosphatidyl-*N*-monomethylethanolamine	1.66 ± 0.05*
Phosphatidyl-*NN*-dimethylethanolamine	1.99 ± 0.09*
Phosphatidylcholine	1.61 ± 0.09*

phosphatidylserine was not due to a contamination with phosphatidylcholine.

Several recent studies have described phospholipase D species that hydrolyse other lipids besides phosphatidylcholine (Balsinde et al., 1988; Kiss and Anderson, 1989; Huang et al., 1992). It is not clear whether the activity against other phospholipids besides phosphatidylcholine represents another isoenzyme of phospholipase D. Wang et al. (1991) have described a cytosolic enzyme from bovine brain that hydrolyses phosphatidylethanolamine better than phosphatidylcholine. Crude cytosolic fractions prepared by their method did not demonstrate any phospholipase D activity in our assay. However, we cannot exclude the possibility that the limited activity against phospholipids other than phosphatidylcholine is mediated by this cytosolic enzyme that is either trapped in the microsomal pellet or loosely bound to the membrane.

In comparable experiments carried out with PC12 cells and primary glial cells (results not shown), phospholipase D was specific for phosphatidylcholine; there was no hydrolysis of the other phospholipid classes. This is consistent with our observations in the intact PC12 cells using dual labelling of phospholipids with [^{14}C]stearic acid and [^{3}H]palmitic acid. Those data indicated that 90% or more of bradykinin-stimulated phosphatidylethanol was derived from phosphatidylcholine (Horwitz, 1991). Thus the cytosolic enzyme described by Wang et al. (1991) does not appear to play a role in mediating the effects of bradykinin on phosphatidylethanol production in the intact cell. Recently, Llahi and Fain (1992) have examined noradrenaline-stimulated phosphatidylethanol production in brain slices whose phospholipids have been prelabelled with [^{32}P]P_i. Since [^{32}P]P_i was incorporated into all the phospholipids, they could all have potentially served as substrates. However, in these studies the decrease in the label in any particular phospholipid was too small to detect. The specificity of neurotransmitter-activated phospholipase D in brain slices will require further investigation.

In animal tissues, phosphatidylcholine can be synthesized by two different pathways. In one pathway the enzyme phosphocholine transferase converts CDP-choline plus diacylglycerol to phosphatidylcholine plus CMP. The other pathway involves the direct methylation of the amino group of phosphatidylethanolamine with *S*-adenosylmethionine as the methyl donor. The intermediates formed upon subsequent methylations are phosphatidyl-*N*-methylethanolamine and phosphatidyl-*N*-dimethylethanolamine. The experiment shown in Table 2 was designed to identify the point at which these phospholipids become good substrates for phospholipase D. In order to exclude the possible influence of fatty acid composition, this experiment was carried out with phospholipids that contain palmitic acid in both the 1 and 2 positions. There was a small but significant activity against phosphatidylethanolamine. Phosphatidyl-*N*-methylethanolamine appears to be a good substrate for phospholipase D. Apparently, the addition of one methyl group converts phosphatidylethanolamine into a good substrate; the specificity for the choline headgroup is, therefore, not absolute.

The physiological significance of the fact that phospholipase D hydrolyses phosphatidyl-*N*-monomethylethanolamine is not clear. The specific activities of the methyltransferases from brain are much lower than those from rat liver (Vance and Ridgway, 1988). In addition, Percey et al. (1982) have estimated, based on the specific activities of choline phosphotransferase and phosphatidylethanolamine methyltransferase in brain, that 1% of brain phosphatidylcholine is synthesized by the methylation pathway. Thus the actual levels of this metabolite must be quite low. On the other hand, several neurotransmitters, including β-adrenergic receptor agonists, histamine and dopamine, have been shown to stimulate methylation in neuronal tissue (Leprohon et al., 1983; Ozawa and Segawa, 1988; Andriamampandry et al., 1991). This methylation is thought to occur via two methylase enzymes, one present on the cytoplasmic side which catalyses the first methylation and the other present on the outside of the cell which catalyses the second and third methylations leading to the formation of phosphatidylcholine (Hirata et al., 1978; Crews et al., 1980). Thus the phosphatidyl-*N*-methylethanolamine that is formed upon stimulation would be present on the cytoplasmic side and, therefore, susceptible to hydrolysis by phospholipase D.

It is apparent that the main substrate for phospholipase D is phosphatidylcholine; however, there is also a relatively small activity against phosphatidylethanolamine and phosphatidylserine. Phosphatidylcholine constitutes 30–50% of total cellular phospholipids. Phosphatidylethanolamine is also a major phospholipid, constituting 15–25% of cellular phospholipids, i.e. about half of the phosphatidylcholine content. However, if phosphatidylcholine is on the outer leaflet of the membrane as has been suggested, there may be some cellular compartments in which there is more phosphatidylethanolamine than phosphatidylcholine (Op den Kamp, 1979). Phosphatidylethanolamine, by being a poor substrate for phospholipase D, could inhibit the hydrolysis of phosphatidylcholine. Table 3 shows that at the maximal concentration of phosphatidylcholine, 2 mM, the combined addition of phosphatidylethanolamine actually inhibited phosphatidylbutanol production. These data suggest the possibility that phosphatidylcholine hydrolysis may be attenuated in some lipid environments depending on the phospholipid composition. A similar type of lipid interaction has been reported for phosphoinositide-specific phospholipase C. Phosphatidylinositol hydrolysis in small unilamellar vesicles was inhibited by phosphatidylcholine (Hofmann and Majerus, 1982). In PC12 cells, the addition of ethanol alone does not lead to an increase in phosphatidylethanol production (Horwitz, 1991). This suggests that basal phospholipase D activity may be attenuated, and a possible cause of this is the particular composition of phospholipids on the cytoplasmic side of the membrane.

The conclusion that phospholipase D is specific for phosphatidylcholine in intact tissue is based primarily on the fatty acid composition of phosphatidylethanol. If phospholipase D is specific for phospholipids of a particular fatty acid composition rather than a particular headgroup, this would not be apparent

Table 3 Additivity of phospholipase D activity against phosphatidylcholine and phosphatidylethanolamine in microsomal membranes

Dipalmitoyl-phosphatidylcholine (final concentration 2 mM) and/or phosphatidylethanolamine (final concentration 1 mM) were dried down under a stream of nitrogen and resuspended in 36 mM sodium oleate that had been warmed to 55 °C. Phospholipids were sonicated in this warm solution and maintained at this temperature until they were added to the assay. The final sodium oleate concentration was 6 mM. All other assay conditions were as described in the Experimental section. Each assay contained 5 μCi of [^{3}H]butanol. Results are means ± S.E.M. of triplicate determinations. The results are representative of at least two similar experiments. *Significant difference at the $P < 0.05$ level from those conditions containing phosphatidylcholine alone.

	Phospholipase D activity (pmol/mg of protein)	
	Control	Phosphatidylethanolamine
No additions	0.04 ± 0.01	0.25 ± 0.03
Phosphatidylcholine	1.38 ± 0.09	0.91 ± 0.08*

Table 4 Specificity of phospholipase D for fatty acid composition

Phospholipid analogues were dried down under a stream of nitrogen and resuspended in sodium oleate, such that the final concentration was 6 mM; phospholipase D was assayed as described in the Experimental section. Each assay contained 5 μCi of [^{3}H]butanol and phospholipid at a concentration of 2 mM. Results are means ± S.E.M. of triplicate determinations. The results are representative of at least two similar experiments. *Significant difference from the no phospholipid control at the $P < 0.05$ level.

	Phosphatidyl-[^{3}H]butanol (mol/mg of protein)
No phospholipid	0.06 ± 0.02
Phosphatidylcholine (β-arachidonyl, γ-stearoyl)	0.66 ± 0.02*
Phosphatidylcholine (β-oleoyl, γ-stearoyl)	1.09 ± 0.02*
Phosphatidylcholine (β-oleoyl, γ-palmitic)	1.16 ± 0.02*
Phosphatidylcholine (egg yolk)	1.77 ± 0.06*

from simply examining the fatty acid composition of the product. The experiment shown in Table 4 was designed to investigate this issue. The two most abundant fatty acids in position 1 of phospholipids are palmitic and stearic acids (Pessin et al., 1991). Phosphatidylcholine contains more palmitic than stearic acid, whereas the other phospholipids contain more stearic than palmitic acid. These two fatty acids were used to dual-label PC12 cells (Horwitz, 1991). In those experiments, the cells were incubated with [^{3}H]palmitic acid and [^{14}C]stearic acid. Bradykinin-stimulated phosphatidylethanol in PC12 cells contained a palmitic acid/stearic acid ratio that was comparable with that in phosphatidylcholine. Since phosphatidylethanol is a specific product of phospholipase D activity, this suggested that phospholipase D was specific for phosphatidylcholine. The experiment shown in Table 4 suggests that this result is not due to a preference of phospholipase D for phospholipids containing palmitic acid. Rather, the specificity is determined by the headgroup.

Although phosphatidylinositol is known to contain a high proportion of arachidonic acid in the 2-position, there are also some molecular species of phosphatidylcholine that contain arachidonic acid in this position. These species as a proportion of the total phosphatidylcholine in the cell may be small; however, the absolute amount may be large compared with that in phosphatidylinositol. By the same reasoning, phosphatidylcholine contains a high proportion of oleic acid in the 2-position, but there are also some minor species of phosphatidylinositol that contain this fatty acid. Phosphatidylcholine with arachidonic acid in the 2-position is not a good substrate for phospholipase D compared with when oleic acid is in the 2-position (Table 4). These results are consistent with those of Mohn et al. (1992). Bagaut et al. (1985) have shown that an alkyl substitution at the 2-position of phosphatidylcholine affects its ability to act as a substrate for cabbage phospholipase D. The fatty acid in the 2-position may affect the ability of phospholipase D to interact with the headgroup. We cannot exclude the possibility that the different fatty acid compositions affect vesicle architecture.

The decrease in activity of phospholipase D when arachidonic acid is in the 2-position might provide a basis for the lack of activity of this enzyme against phosphatidylinositol. We examined this issue further by measuring the ability of phosphatidylinositols of different fatty acid compositions to act as a substrate for phospholipase D. Phosphatidylinositol from soybean contains a high level of oleic acid, while that isolated from liver contains a high level of arachidonic acid (Myher and Kuksis, 1984). There was no difference in phospholipase D activities when these lipids were added to the assay as substrates (control, 0.06 ± 0.02; soybean, 0.33 ± 0.05; liver, 0.29 ± 0.08 pmol/mg of protein). Thus the specificity of phospholipase D is determined primarily by the headgroup.

The results presented here confirm and expand upon the previous reports of the specificity of neuronal phospholipase D. The specificity of phospholipase D for phosphatidylcholine is determined by the headgroup; fatty acid composition does not play a role. In addition, the phospholipid environment may also influence the rate of hydrolysis. The production of phosphatidyl[^{3}H]butanol from an exogenous phospholipid substrate appears to be a reliable and versatile assay for phospholipase D activity. This assay should prove to be useful in other applications, such as purification of the enzyme.

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REFERENCES

Andriamampandry, C., Freysz, Ll, Kanfer, J. N., Dreyfus, H. and Massarelli, R. (1991) J. Neurochem. **56**, 1845–1850
Bagaut, M., Kuksis, A. and Myher, J. J. (1985) Biochim. Biophys. Acta **835**, 304–314
Balsinde, J., Diez, E. and Mollinedo, F. (1988) Biochem. Biophys. Res. Commun. **154**, 502–508
Banerjee, P., Buse, J. T. and Dawson, G. (1990) Biochim. Biophys. Acta **1044**, 305–314
Besterman, J. M., Duronio, V. and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6785–6789
Billah, M. M. and Anthes, J. C. (1990) Biochem. J. **269**, 281–291
Bocckino, S. B., Blackmore, P. F. and Exton, J. H. (1985) J. Biol. Chem. **260**, 14201–14207
Bocckino, S. B., Blackmore, P. F., Wilson, P. B. and Exton, J. H. (1987) J. Biol. Chem. **262**, 15309–15315
Chalifa, V., Mohn, H. and Liscovitch, M. (1990) J. Biol. Chem. **265**, 17512–17519
Crews, F. T., Hirata, F. and Axelrod, J. (1980) J. Neurochem. **34**, 1491–1498
Exton, J. H. (1990) J. Biol. Chem. **265**, 1–4
Hirata, F., Viveros, O. H., Diliberto, E. J., Jr. and Axelrod, J. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 1718–1721
Hofmann, S. L. and Majerus, P. W. (1982) J. Biol. Chem. **257**, 14359–14364
Holbrook, P. G., Pannell, L. K., Mirata, Y. and Daly, J. W. (1992) J. Biol. Chem. **267**, 16834–16840
Horwitz, J. (1990) J. Neurochem. **54**, 983–991

Horwitz, J. (1991) J. Neurochem. **56**, 509–517
Huang, C., Wykle, R. L., Daniel, L. W. and Cabot, M. C. (1992) J. Biol. Chem. **267**, 16859–16865
Hughes, B. P., Rye, K.-A., Pickford, L. B., Barritt, G. J. and Chalmers, A. H. (1984) Biochem. J. **222**, 535–540
Kiss, Z. and Anderson, W. B. (1989) J. Biol. Chem. **264**, 1483–1487
Kobayashi, M. and Kanfer, J. N. (1987). J. Neurochem. **48**, 1597–1603
Leprohon, C. E., Blusztajn, J. K. and Wurtmman, R. J. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 2063–2066
Liscovitch, M. (1989) J. Biol. Chem. **264**, 1450–1456
Llahi, S. and Fain, J. N. (1992) J. Biol. Chem. **267**, 3679–3685
Mohn, H., Chalifa, V. and Liscovitch, M. (1992) J. Biol. Chem. **267**, 11131–11136
Myher, J. J. and Kuksis, A. (1984) Biochim. Biophys. Acta **795**, 85–90
Op den Kamp, J. A. F. (1979) Annu. Rev. Biochem. **48**, 47–71
Ozawa, K. and Segawa, T. (1988) J. Neurochem. **50**, 1551–1558
Percey, A. K., Moore, J. F. and Waechter, C. J. (1982) J. Neurochem. **38**, 1404–1412

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Pessin, M. S. and Raben, D. M. (1989) J. Biol. Chem. **264**, 8729–8738
Pessin, M. S., Altin, J. G., Jarpe, M., Tansley, F., Bradshaw, R. A. and Raben, D. M. (1991) Cell Regul. **2**, 383–390
Ragab-Thomas, J. M., Hullin, F., Chap, H. and Douste-Blazy, L. (1987) Biochim. Biophys. Acta **917**, 388–397
Randall, R. W., Bonser, R. W., Thompson, N. T. and Garland, L. G. (1990) FEBS Lett. **264**, 87–90
Rosoff, P. M., Savage, N. and Dinarello, C. A. (1988) Cell **54**, 73–81
Shukla, S. D. and Halenda, S. P. (1991) Life Sci. **48**, 851–866
Takamura, H., Narita, H., Park, H. J., Tanaka, K., Matsuura, T. and Kito, M. (1987) J. Biol. Chem. **262**, 2262–2269
Traynor, A. E., Schubert, D. and Allen, W. R. (1982) J. Neurochem. **39**, 1677–1683
Vance, D. E. and Ridgway, N. D. (1988) Prog. Lipid Res. **27**, 61–79
Wang, P., Anthes, J. C., Siegel, M. I., Egan, R. W. and Billah, M. M. (1991) J. Biol. Chem. **266**, 14877–14880
Witter, B. and Kanfer, J. N. (1985) J. Neurochem. **44**, 155–162