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Alcohols selectively stimulate phospholipase D-mediated hydrolysis of phosphatidylethanolamine in NIH 3T3 cells

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Addition of alcohols to NIH 3T3 fibroblasts, prelabeled with [2-^{14}C]ethanolamine, resulted in increased degradation of [^{14}C]phosphatidylethanolamine (PtdEtn). Long-chain alcohols, like octanol or nonanol, were more potent than methanol or ethanol. The main water-soluble product of alcohol-stimulated [^{14}C]PtdEtn hydrolysis was [^{14}C]ethanolamine. Addition of ethanol to cells, specifically prelabeled with [^{32}P]PtdEtn, enhanced the formation of [^{32}P]phosphatidic acid (PtdOH), suggesting the involvement of a phospholipase D-type enzyme. At lower concentrations (10–150 mM), ethanol acted through a protein kinase C (PKC)-independent mechanism. At higher concentrations (150–300 mM), the effect of ethanol was partially inhibited both by the PKC inhibitor H7 and by the down-regulation of PKC achieved by treatment of cells with 200 nM TPA for 24 h, suggesting that activation of PKC contributed to the ethanol effect.

Alcohol; Phospholipase D; Phosphatidylethanolamine hydrolysis

1. INTRODUCTION

Interaction of ethanol with the cell membrane results in the activation of basic signal transduction systems. Thus, ethanol was shown to stimulate both the cyclic AMP-generating adenylate cyclase system [1–4], and phospholipase C [5,6]. In the latter case, phospholipase C-catalyzed hydrolysis of polyphosphoinositides is associated with the formation of two intracellular messengers, inositol 1,4,5-trisphosphate and DAG. While inositol 1,4,5-trisphosphate stimulates the release of calcium from intracellular stores [7], DAG stimulates PKC [8].

Here we show that in NIH 3T3 fibroblasts ethanol and other alcohols also stimulated the hydrolysis of PtdEtn, but not that of PtdCho. Since PtdOH, one of the primary breakdown products of alcohol-stimulated phospholipid hydrolysis, has growth factor-like effects [9,10] as well as other biological activities [11,12] in these cells, increased PtdEtn hydrolysis may be part of the signal transduction system, and may be related to some of the physiological actions of ethanol.

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PtdEtn, phósphatidylethanolamine; PtdCho, phosphatidylcholine; PtdEt, phosphatidylethanol; PtdOH, phosphatidic acid; DAG, 1,2-diacylglycerol; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine

2. MATERIALS AND METHODS

2.1. *Materials*

TPA, PtdEtn (type I), lysophosphatidylethanolamine, PtdCho, and phospholipase D (cabbage) were purchased from Sigma; ethanol, methanol, and glycerol were from Baker, the other alcohols were from Sigma; [2-^{14}C]ethanolamine (20 mCi/mmol), [*methyl*-^{14}C]choline chloride (50 mCi/mmol), and [^{32}P]orthophosphate (carrier-free in water) were from Amersham. PtdEt was prepared by a phospholipase D-catalyzed reaction from PtdCho and ethanol [13]. [^{32}P]PtdEtn (185 mCi/mmol) was prepared by incubating beans with [^{32}P]orthophosphoric acid for 48 h, followed by the isolation of this phospholipid by thin-layer chromatography.

2.2. *Cell culture*

NIH 3T3 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, penicillin-streptomycin (50 U/ml and 50 μg/ml, respectively), and glutamine (2 mM). For all experimental studies the cells were used during the exponential phase of growth.

2.3. *Measurement of alcohol-stimulated hydrolysis of PtdEtn*

NIH 3T3 cells wre incubated with [2-^{14}C]ethanolamine (0.5 μCi/ml) for 48 h, washed, incubated for 3 h in the presence of fresh medium, and then harvested by gently scraping from the dish. Collected cells, pooled from 3–5 dishes (150 cm^2), were washed. Then 0.3 ml aliquots of this cell suspension (1.4 to 3.6 $\times$ 10^6 cells/ml) were incubated (final volume 0.35 ml) in polypropylene tubes in an incubator for 0–2 h in the presence of 2 mM ethanolamine, and in the absence or presence of alcohols as indicated. Incubations were terminated by the addition of 4 ml chloroform/methanol (1 : 1, v/v). After phase separation, initiated with 1 M KCl (2.2 ml), the ^{14}C content was determined in 1.5 ml aliquots of the upper phase using a liquid scintillation spectrophotometer. The lower (chloroform) phase was evaporated under N_2, redissolved in 50 μl chloroform, and phospholipids were separated by silica gel thin layer chromatography [14,15]. Distribution of ^{14}C activity among the water-soluble products of [^{14}C]ethanolamine in the residue remaining after evaporation of the upper phase was determined as in [16].

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For the determination of alcohol effect on the degradation of cellular [^{32}P]PtdEtn, cells were first incubated with [^{32}P]PtdEtn-containing liposomes (1.25 μCi/ml) for 48 h. Liposomes were prepared by sonication of a mixture of [^{32}P]PtdEtn/PtdCho/phosphatidylserine (1:3:2, molar ratio). After the labeling period, non-incorporated radioactivity was removed by thorough washing of cells. Cells were harvested by scraping as above and incubated in the absence or presence of ethanol (150 mM) for 1 h. In each case, the incubation medium also contained 0.3 mM propranolol to inhibit the activity of PtdOH phosphohydrolyse [17–19].

For the determination of PtdCho degradation, cells were prelabeled with [*methyl*-^{14}C]choline (0.5 μCi/ml) for 48 h, washed, incubated for 3 h in fresh medium, then treated with 10–300 mM concentrations of alcohols and/or 100 nM TPA for 2 h in the presence of 25 mM unlabeled choline. Further steps were as described above.

2.4. *Determination of phosphatidylethanol synthesis*

Cells were prelabeled with [1-^{14}C]palmitic acid (0.25 μCi/ml) for 8 h, washed twice with 10 ml of fresh medium, and incubated for an additional 2 h to diminish the cellular content of nonesterified [^{14}C]palmitic acid. Cells were harvested by scraping from the dish, washed, and then reincubated (2×10^6 cells/ml) for 30–120 min in the absence or presence of alcohols (10–300 mM) and/or TPA (100 nM). Incubations were terminated by the addition of chloroform/methanol (1:1, v/v), and after addition of PtdEt standard (30 μg/sample) phospholipids were separated on silica gel G plates as in [13].

3. RESULTS AND DISCUSSION

Addition of ethanol or other alcohols to [^{14}C]ethanolamine-prelabeled NIH 3T3 cells stimulated the release of water-soluble ^{14}C activity from the phospholipid pool. The maximally effective concentrations of alcohols, along with the rate of stimulation achieved, are shown in table 1. Evidently, octanol and nonanol were more potent stimulators of PtdEtn degradation than short-chain alcohols. In each case, formation of water-soluble [^{14}C]ethanolamine products was accompanied by a similar loss of [^{14}C]PtdEtn (data not shown). Although maximal effects required high concentrations, significant effects also were observed at physiologically relevant alcohol concentrations. For example, 25 and 50 mM concentrations of ethanol stimulated the hydrolysis of PtdEtn 22 and 32%, respectively.

In principle, the alcohol-induced formation of water-soluble [^{14}C]ethanolamine products could occur through a number of distinct mechanisms, including (i) the formation of PtdEt from PtdEtn and alcohols by a transphosphatidylation mechanism, (ii) the possible stimulation of exchange of phospholipid-bound ethanolamine with choline or serine, (iii) the sequential deacylation of PtdEtn by phospholipase A-type enzymes leading to the formation of glycerophosphoethanolamine, or (iv) the action of a phospholipase C- or D-type enzyme. Next, all these possibilities were examined.

To evaluate the role of the transphosphatidylation mechanism, we also studied the effect of ethanol on the hydrolysis of PtdCho, the principal substrate for PtdEt synthesis [13]. Ethanol or other alcohols had no stimulatory effects on [^{14}C]PtdCho hydrolysis, studied in [^{14}C]choline-prelabeled cells (table 1). We should note here that in other cellular systems [20,21] no PtdEt synthesis was observed in the absence of TPA, an activator of PKC [8]. Similarly, in these cells we observed alcohol-induced loss of [^{14}C]PtdCho and a concomitant formation of PtdEt only in the copresence of TPA (data not shown). In addition, we were unable to demonstrate phosphatidylalcohol formation with octanol or nonanol as substrates even in the presence of TPA (data not shown). For these reasons, a transphosphatidylation mechanism did not seem to be involved in the alcohol-induced degradation of PtdEtn.

To probe the possible role of the base-exchange mechanism, 20 mM choline or serine were added to [^{14}C]ethanolamine-prelabeled cells in the absence or

Table 1

Effect of alcohols on the degradation of ^{14}C-labeled phospholipids in NIH 3T3 cells

Addition	Degradation of ^{14}C-labeled phospholipids (dpm/10^6 cells per 2 h chase)	
	[^{14}C]Ethanolamine products	[^{14}C]Choline products
None	16850 ± 730	4800 ± 420
Methanol (400 mM)	29310 ± 860	4640 ± 390
Ethanol (300 mM)	32030 ± 610	3780 ± 640
Glycerol (150 mM)	30990 ± 620	3920 ± 330
1-Pentanol (50 mM)	34660 ± 640	–
1-Hexanol (50 mM)	36390 ± 410	4060 ± 290
1-Octanol (25 mM)	46220 ± 1140	–
1-Nonanol (25 mM)	49450 ± 970	4140 ± 460

Cells were prelabeled with either [^{14}C]ethanolamine or [^{14}C]choline for 48 h, followed by treatments with various concentrations (1–500 mM) of alcohols for 2 h (chase period). Released water-soluble ^{14}C products were measured as described in section 2. Values obtained with maximally effective concentrations are given. Data are the mean ± SE of four determinations. Similar results were obtained in two other experiments

Table 2

Distribution of ^{14}C activity among water-soluble ethanolamine products derived from ethanol-stimulated hydrolysis of phosphatidylethanolamine

Addition	^{14}C label in water-soluble ethanolamine products (dpm/10^6 cells per 2 h chase)			
	Total amine	Ethanol-amine	Phospho-ethanol	X
None (control)	13240 ± 810	6920 ± 300	3760 ± 720	940 ± 180
Ethanol (300 mM)	24980 ± 840	17760 ± 1260	2640 ± 340	3040 ± 100

Cells were prelabeled with [^{14}C]ethanolamine for 48 h, treated with ethanol for 2 h (chase period), and the incorporation of ^{14}C into various isolated water-soluble ethanolamine products was determined. Data are the mean ± SE of three determinations

Table 3

Stimulation by ethanol of [^{32}P]phosphatidic acid formation from [^{32}P]PtdEtn in NIH 3T3 cells

Addition	Incubation time (min)	^{32}P label (dpm/10^6 cells per 60 min)				
		PtdOH	PtdEtn	Lyso-PtdEtn	PtdCho	Water-soluble fraction
None	0	179 ± 27	8820 ± 270	1206 ± 88	1047 ± 54	31 ± 17
None	60	128 ± 32	8290 ± 130	763 ± 24	925 ± 22	188 ± 39
Ethanol	60	337 ± 41	7610 ± 75	786 ± 51	885 ± 13	211 ± 46

Cells, prelabeled with [^{32}P]PtdEtn as described in section 2, were incubated in the absence or presence of 150 mM ethanol for 60 min. Data are the mean ± SE of three determinations

presence of ethanol. These compounds, capable of replacing phospholipid-bound ethanolamine [22], did not modify the alcohol effect on PtdEtn hydrolysis, indicating that a base-exchange mechanism was not responsible for alcohol-induced hydrolysis of PtdEtn (data not shown).

To further characterize the mechanism involved in the alcohol-induced degradation of [^{14}C]PtdEtn, the water-soluble products were separated by thin layer chromatography. As shown in table 2, most water-soluble ^{14}C-labeled material was found to comigrate with the ethanolamine standard which is compatible with the involvement of phospholipase D in the alcohol-stimulated hydrolysis of PtdEtn. It should be noted that ethanol also stimulated the formation of an ethanolamine derivative, designated X in table 2, which comigrated with CDP-ethanolamine on silica gel plates, but which clearly migrated to a distinct position on PEI-cellulose plates with 0.1 M LiCl as solvent. Identification of this unknown compound is in progress.

Alcohols failed to increase the cellular content of [^{14}C]lyso-PtdEtn (data not shown). This, however, did not rule out the possibility that increased degradation of PtdEtn by sequential deacylation, leading to the formation of glycerophosphoethanolamine, was involved in the alcohol effect. Clearly, understanding of the mechanism of alcohol action required selective utilization of ^{32}P-labeled PtdEtn to determine whether alcohols increased the formation of [^{32}P]PtdOH or [^{32}P]glycerophosphoethanolamine. For this purpose, cells prelabeled with [^{32}P]PtdEtn were used. Although the uptake of this phospholipid by cells was an inefficient process, it provided the necessary specificity. Thus, of the total ^{32}P activity taken up by the cells about 80% was associated with PtdEtn, with the rest of radioactivity being almost equally distributed between PtdCho and lyso-PtdEtn. As shown in table 3, ethanol stimulated the degradation of [^{32}P]PtdEtn, but it failed to enhance the formation of [^{32}P]lyso-PtdEtn. Similarly, ethanol had no effect on the formation of ^{32}P-labeled water-soluble products. On the other hand, ethanol clearly enhanced the level of [^{32}P]PtdOH (table 3). Since ethanol did not stimulate the hydrolysis of [^{32}P]PtdCho, formed by the methylation of [^{32}P]PtdEtn, this latter phospholipid was the only possible source of [^{32}P]PtdOH formed in the presence of ethanol. We should note that even in the presence of propranolol, a powerful inhibitor of PtdOH phosphohydrolyse [17–19], only about 30% of hydrolyzed [^{32}P]PtdEtn was recovered in [^{32}P]PtdOH. The most likely possibility is that the major portion of PtdOH was used for phosphatidylinositol synthesis, a mechanism which would bypass the phosphohydrolyse enzyme.

Next, we studied the possibility that the alcohol effect involved the activation of PKC. First, for a nearly complete down-regulation of PKC [23–25], TPA (200 nM) was added to cells, prior to ethanol treatment, for the last 24 h of the ^{14}C-labeling period. As indicated in table 4, prolonged treatment of cells with TPA completely blocked the effect of subsequently added TPA on PtdEtn hydrolysis. H7, an inhibitor of PKC [26], also significantly (63%) inhibited the effect

Table 4

Effect of ethanol and TPA on the degradation of [^{14}C]PtdEtn in control, TPA-pretreated, and H7-treated NIH 3T3 cells

Addition	Degradation of [^{14}C]PtdEtn to [^{14}C]ethanolamine (dpm/10^6 cells per 2 h chase)		
	Untreated	TPA-pretreated	+H7 (300 μM)
None	16280 ± 290	17060 ± 690	16890 ± 530
TPA (100 nM)	39590 ± 540	17750 ± 270	26630 ± 280
Ethanol (150 mM)	23400 ± 380	22510 ± 420	22280 ± 460
Ethanol (300 mM)	31920 ± 770	23220 ± 470	24660 ± 370

Cells were prelabeled with [^{14}C]ethanolamine for 48 h. Where indicated, 200 nM TPA was added for the last 24 h of the prelabeling period (TPA-pretreated cells). Prelabeled cells were further incubated for 2 h (chase period) in the presence of the agents indicated. Data are the mean ± SE of four determinations. Similar results were obtained in two other experiments

of TPA, further indicating the involvement of PKC. Interestingly, pretreatment of cells with TPA, or addition of H7 did not modify the stimulatory effect of 150 mM ethanol. In contrast, these treatments significantly inhibited the stimulatory effect of 300 mM ethanol (table 4).

We have reported earlier that the stimulatory effect of TPA on PtdEtn hydrolysis occurred only after a 10 min lag period [16]. Presently, alcohols had significant effects only after a 20–30 min lag period (data not shown).

In conclusion, available evidence suggests that in NIH 3T3 cells alcohols specifically stimulate the degradation of PtdEtn, but not that of PtdCho. Degradation of PtdEtn resulted in the formation of ethanolamine and PtdOH, suggesting that a phospholipase D-type enzyme was involved. However, alcohols failed to stimulate the hydrolysis of PtdCho, the most preferred substrate of phospholipase D [27]; the reason for this is presently not understood. One possibility is that in these cells multiple forms of phospholipase D exist, each interacting with specific phospholipids and responding differently to various regulatory agents. Another possibility is that the specific effects of alcohols reflect asymmetric distribution of phospholipids and/or alcohols in the plasma membrane. Finally, alcohols might have increased the formation of PtdOH and ethanolamine through more complex, presently unknown, mechanisms. Irrespective of the mechanism of ethanol action, increased degradation of PtdEtn resulted in increased formation of PtdOH, a biologically very potent compound [8–12]. At physiologically relevant concentrations ethanol-stimulated PtdEtn hydrolysis did not involve PKC. At nonphysiologically high concentrations, however, the alcohol effect involved the activation of PKC. The slow onset of alcohol action would be consistent with an indirect mechanism. The specific stimulation of PtdEtn hydrolysis by alcohols may not be an isolated phenomenon. Recently, Kester et al. [28] reported that interleukin-1 specifically stimulated PtdEtn degradation in cultured mesangial cells. We are presently studying the possibility that alcohols potentiated the effect of interleukin-1 or a similarly potent compound.

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