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Ca^{2+}-mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation

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Vasopressin, angiotensin II and epinephrine elicited the accumulation of phosphatidylethanol in rat hepatocytes exposed to ethanol and of phosphatidate in the absence of ethanol. When isolated liver plasma membranes were exposed to ethanol, GTPγS stimulated the production of phosphatidylethanol whereas phosphatidate was formed in the absence of ethanol. With increasing ethanol concentrations, phosphatidate formation declined whereas phosphatidylethanol production increased. These findings suggest that rat hepatocytes possess a hormone-dependent phospholipase D activity that can also catalyze the formation of phosphatidylethanol.

Phosphatidylethanol; Phospholipase D; Ca^{2+}-mobilizing hormone; Ethanol; Phosphatidic acid; (Rat liver)

1. INTRODUCTION

Recently, Alling and co-workers [1,2] discovered an abnormal lipid, phosphatidylethanol, in the tissues of ethanol-intoxicated rats. This lipid can be formed by cabbage phospholipase D through a transphosphatidylation reaction in which ethanol substitutes for water as the phosphatidyl acceptor [3]. Rat brain phospholipase D has also been shown to catalyze the formation of phosphatidylethanol from phosphatidylcholine and ethanol; this reaction is stimulated by millimolar concentrations of oleate, an activator of brain phospholipase D [4,5].

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Abbreviation: GTPγS, guanosine 5'-(3-*O*-thio)triphosphate

We have recently described a phospholipase D activity in rat hepatocytes that is stimulated by calcium-mobilizing hormones apparently via a G-protein [6]. Here, we show that calcium-mobilizing hormones elicit the formation of phosphatidylethanol in hepatocytes treated with ethanol, and that this compound is also produced by liver plasma membranes treated with GTPγS and ethanol. The formation of phosphatidylethanol in tissues may provide an indirect, but simple, index of phospholipase D activity.

2. MATERIALS AND METHODS

2.1. *Materials*

Phosphatidic acid (dipalmitoyl) and phosphatidylcholine (bovine liver) were from Avanti (Birmingham, AL). Epinephrine [8-arginine]vasopressin, angiotensin II, phospholipase D (cabbage), Hepes, EGTA and the ethanol assay kit (322-A) were from Sigma; ethyl acetate, chloroform and methanol from American Burdick and Jackson; iso-octane and acetic acid from Fisher

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Scientific; ethyl alcohol USP from Aaper (Shelbyville, KY), and GTPγS from Boehringer Mannheim. Phosphatidylethanol standard was prepared from bovine liver phosphatidylcholine as described by Kobayashi and Kanfer [4] and purified by thin-layer chromatography as described below.

2.2. *Cell incubations*

Hepatocytes were prepared from fed male rats (250–300 g) as in [7]. Hepatocyte suspensions (75–90 mg/ml wet wt) were shaken and gassed with 95% O_2:5% CO_2 for 15 min at 37°C before the addition of ethanol. Hormones were added 20 min after the ethanol. After a further 10 min, 1-ml aliquots were extracted using the method of Bligh and Dyer [8].

2.3. *Membrane incubations*

Rat liver plasma membranes were prepared as described [9] and washed twice by centrifugation with 50 mM Hepes, 1 mM EGTA (pH 7.5). The membranes were resuspended in this buffer at approx. 2 mg protein/ml. The membrane suspension was added to an equal volume of 20 mM $MgCl_2$ and where indicated, GTPγS (final concentration, 20 μM), and ethanol. The mixtures (400 μl volume) were incubated for 10 min at 37°C with shaking and terminated by the addition of 1.5 ml of $CHCl_3$/Me-OH (1:2) followed by $CHCl_3$ (0.5 ml) and H_2O (0.5 ml) [8]. The samples were assayed for phosphatidylethanol and phosphatidate as described below. Protein was assayed using the bicinchoninic acid method (Pierce).

Table 1

Effect of calcium-mobilizing hormones on phosphatidylethanol accumulation in rat hepatocytes

Hormone	+ ethanol (phosphatidylethanol) (pmol/mg wet wt) (± SE)	− ethanol (phosphatidate) (ng/mg wet wt) (± SE)
None	5.8 ± 2.8	57.7 ± 2.7
Vasopressin (10^{-7} M)	50.4 ± 3.1	132.8 ± 2.7
Angiotensin II (10^{-7} M)	33.1 ± 4.0	84.2 ± 0.1
Epinephrine (10^{-5} M)	24.2 ± 2.3	75.9 ± 7.1

Hepatocytes were incubated for 20 min with (phosphatidylethanol) or without (phosphatidic acid) 70 mM EtOH followed by 10 min with the indicated hormone. Phosphatidylethanol and phosphatidate were quantitated as described in section 2. Incubations were performed in triplicate

2.4. *Thin-layer chromatography of lipids*

Phosphatidylethanol and phosphatidic acid were separated from each other and from other lipids by chromatography on 20 × 10 cm silica gel F-254 plates (Merck). Lipid samples equivalent to 15 mg wet wt (hepatocytes) or 0.1 mg protein (membranes) along with phosphatidylethanol and phosphatidate standards were applied in 20 μl $CHCl_3$. The plates were developed with the solvent system ethyl acetate/iso-octane/acetic acid (9:5:2) [10]. The plates were dried, dipped in $CuSO_4$ (5%, w/v), phosphoric acid (4%, w/v), and heated at 190°C for 20 min to visualize the lipids [11]. The lipids were quantitated by densitometry [11].

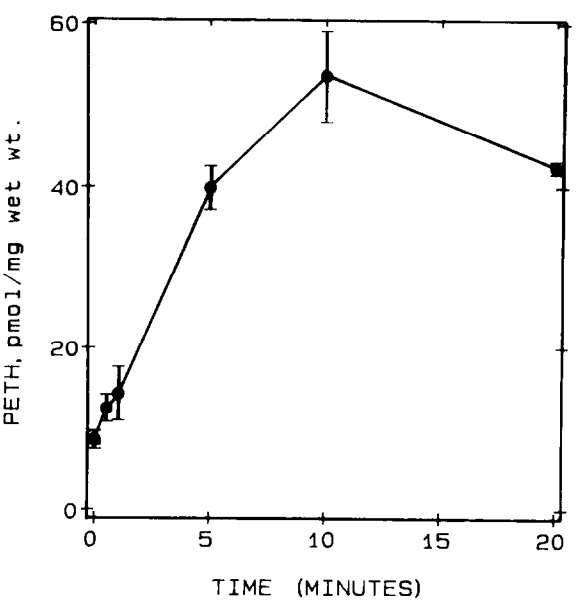


Fig.1. Time course of phosphatidylethanol accumulation in isolated hepatocytes. Vasopressin (0.1 μM) was added (at 0 min) to the hepatocyte suspension after 20 min of incubation with 70 mM ethanol. Aliquots were removed at the indicated times after the addition of vasopressin and assayed for phosphatidylethanol (PETH) as described in section 2. Values represent means ± SE of triplicate incubations.

2.5. *Hydrolysis of phosphatidylethanol with cabbage phospholipase D*

Rat liver membranes (30 mg protein) were incubated with ethanol (200 mM) and GTPγS (20 μM) as above for 15 min at 37°C. The reaction was terminated with $CHCl_3$/MeOH (1:2) and the lipids extracted according to Bligh and Dyer [8]. The extract was evaporated under N_2, resuspended in 500 μl $CHCl_3$ and applied in a streak to a 20 × 20 cm silica gel F-254 plate. A phosphatidylethanol standard was chromatographed adjacent to the membrane lipid. The plate was developed as described above and dried. The lipids were visualized with I_2 vapor and the membrane phosphatidylethanol band was removed. The silica gel was washed with 3 × 3.3 ml $CHCl_3$/MeOH/H_2O (5:10:4). $CHCl_3$ (2.63 ml) and H_2O (2.63 ml) were added with vortex-mixing and the phases were separated by centrifugation. The lower layer was dried under N_2 and the purified membrane phosphatidylethanol was

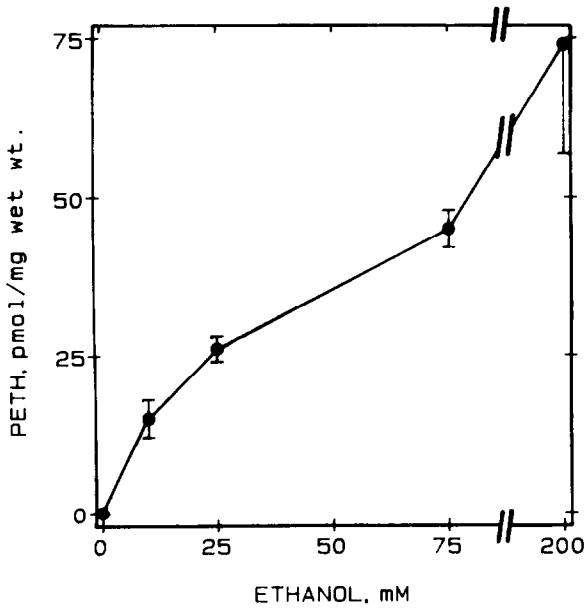
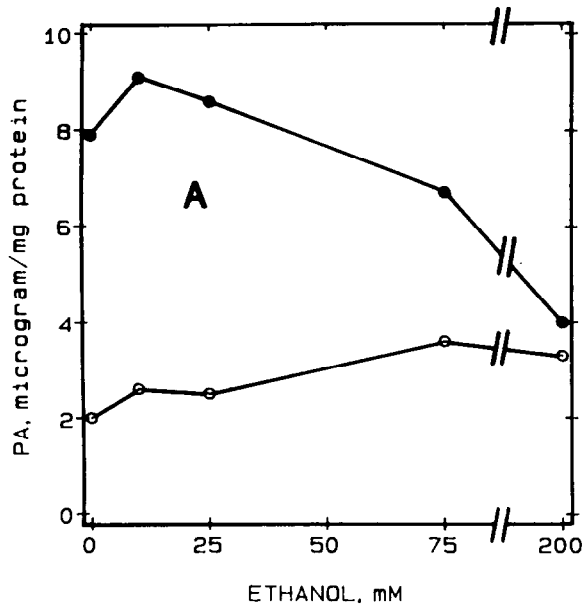


Fig.2. Effect of ethanol concentration on phosphatidylethanol accumulation in the presence of vasopressin. Hepatocytes were incubated with the indicated concentrations of ethanol for 20 min after which vasopressin (0.1 μM) was added. After an additional 10 min of incubation, aliquots were assayed for phosphatidylethanol (PETH) as described in section 2. Values represent means ± SE of triplicate incubations.



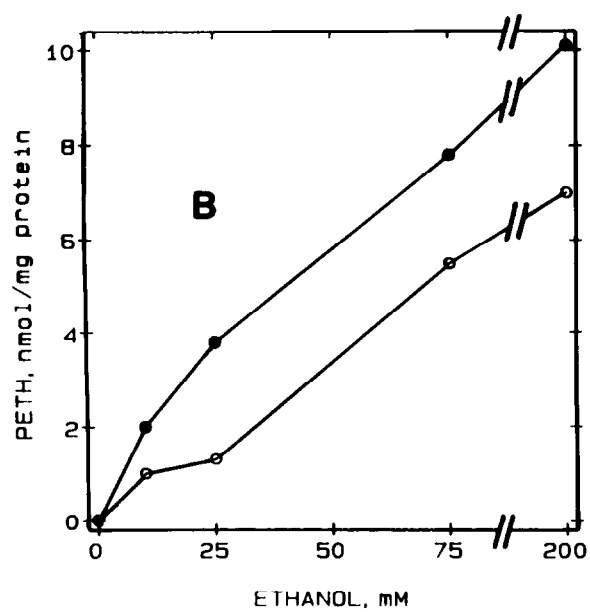


Fig.3. Effect of ethanol concentration on phosphatidylethanol and phosphatidate formation by isolated plasma membranes. Plasma membranes were incubated for 10 min with the indicated amounts of ethanol in the presence (●) or absence (○) of GTPγS (20 μM), and assayed for phosphatidate (PA, panel A) or phosphatidylethanol (PETH, panel B) as described in section 2. Incubations were performed in duplicate.

dissolved in $CHCl_3$. Separate aliquots were assayed for PO_4 [6] and for ethanol after hydrolysis with phospholipase D under the conditions described by Kobayashi and Kanfer [4]. Ethanol was assayed using the alcohol dehydrogenase kit from Sigma.

3. RESULTS AND DISCUSSION

When rat hepatocytes were incubated with ethanol (70 mM), phosphatidylethanol accumulated markedly in response to the calcium-mobilizing hormones, vasopressin, angiotensin II and epinephrine (table 1). In the absence of ethanol these hormones elicited the formation of phosphatidic acid (table 1) which probably arises in part by a phospholipase D mechanism as detailed elsewhere [6]. Phosphatidylethanol production (at 70 mM ethanol) was maximal at 10 min following the addition of vasopressin (0.1 μM) (fig.1) and this time was chosen for the remaining experiments reported here. When cells were incubated with vasopressin (0.1 μM) and varying concentrations of ethanol, phosphatidylethanol accumulated at clinically relevant ethanol concentrations (fig.2).

As shown by us previously [6], rat liver membranes possess a phospholipase D activity which is stimulated by GTPγS, presumably via a G-protein. When rat liver plasma membranes were incubated with GTPγS (20 μM) phosphatidic acid formation was stimulated about 4-fold (fig.3A and [6]). As the concentration of ethanol was increased, GTPγS-dependent phosphatidate production declined (fig.3A) and phosphatidylethanol production increased (fig.3B). Phosphatidylethanol production was increased approx. 2.9-fold by the addition of GTPγS (fig.3B) at 25 mM EtOH. These findings can be interpreted as a competitive situation in which phosphatidylethanol is formed at the expense of phosphatidate by a GTPγS-dependent phospholipase D. This competition has been described for the phospholipase D from rat brain [4].

To confirm that the compound generated in the incubations with membranes and migrating on silica gel plates in the region of the phosphatidylethanol standard was in fact this substance, it was subjected to hydrolysis with phospholipase D and analyzed as described in section 2. A PO_4:EtOH ratio of 0.97 was obtained, confirming its identity.

These results raise the possibility that, in vivo, phosphatidylethanol accumulation in liver may be dependent on calcium-mobilizing hormones. Ethanol has been shown to cause the release of angiotensin II [12] which could in turn elicit phosphatidylethanol formation. The significance of this lipid in liver is unknown, but manipulation of phosphatidylethanol levels by hormones and antagonists may provide some insight.

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