Activity of phosphatidylinositol transfer protein is sensitive to ethanol and membrane curvature

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Phosphatidylinositol transfer protein (PITP) is critical for many cellular signalling and trafficking events that are influenced by ethanol. The influence of ethanol and membrane curvature on the activity of recombinant mouse PITP-α *in vitro* is evaluated by monitoring the transfer of phosphatidylinositol (PtdIns) from rat hepatic microsomes to unilamellar vesicles. Acute exposure to pharmacological levels of ethanol enhanced the function of PITP. Chloroform shared a similar ability to enhance function when both drug concentrations were normalized to their respective octanol/water partition coefficients, indicating that the effect is not unique to ethanol and might be common to hydrophobic solutes. Neither the PITP activity nor its ethanol enhancement was altered by using thermally pretreated (denatured) or protease-treated microsomes, indicating that the native microsomal protein structure was unlikely to be a determinant of transfer. Kinetic analyses indicated that ethanol acted by increasing the PITP-mediated flux of PtdIns from both microsomal and liposomal surfaces. The activity of PITP was strongly dependent on the lipid structure, with a steep dependence on the expressed curvature of the membrane. Activity was greatest for small, highly curved sonicated vesicles and decreased markedly for large, locally planar unilamellar vesicles. Ethanol enhanced PITP-mediated PtdIns transfer to all vesicles, but its effect was much smaller than the enhancement due to curvature, which is consistent with ethanol's comparatively modest ability to perturb membrane lipids. The ethanol efficacy observed is as pronounced as any previously described lipid-mediated ethanol action. In addition, these observations raise the possibility that PITP specifically delivers PtdIns to metabolically active membrane domains of convex curvature and/or low surface densities of lipid.

Key words: anaesthetic, membrane curvature, membrane fluidity, polyphosphoinositide signalling, signal transduction.

INTRODUCTION

Alcohol abuse is linked to numerous disorders in many organs, including the liver, brain, heart, skeletal muscle and pancreas [1]. Ethanol administered acutely and chronically is known to interfere with polyphosphoinositide signalling pathways [2,3]. The acute administration of ethanol is known to disorder or fluidize membranes. Chronic exposure to ethanol induces an adaptive change in membrane properties known as membrane tolerance that is manifested by a resistance to the disordering effects of ethanol [4]. The resistance to disordering was retained in liposomes prepared from the phospholipid extracts [5]. Of the microsomal phospholipid fractions, the ability to resist the disordering effects of ethanol was accentuated in the phosphatidylinositol fraction [5,6].

In intact hepatocytes, ethanol and other short-chain alcohols activated the polyphosphoinositide-specific phospholipase C and initiated the polyphosphoinositide signal transduction cascade [2,3]. In permeabilized hepatocytes (under conditions in which hormonal stimulation was abolished), ethanol was ineffective in activating G-protein-mediated phospholipase Cβ [7]. In platelets the activation of the G-protein-mediated phospholipase Cβ was sensitive to the degree of permeabilization [8]. These studies raised the possibility that a cytosolic cofactor might be an important element in the response to ethanol. It was shown subsequently that controlled permeabilization of HL60 cells permitted the release of endogenous phosphatidylinositol transfer protein (PITP) while maintaining polyphosphoinositide-specific phospholipase Cβ levels, but that PITP was critical for activation of the signal transduction cascade [9]. These results suggested that PITP might be the cofactor required for activation by ethanol. Unlike most receptor-mediated agonist activators of the signal cascade, ethanol elevated levels of the phosphorylated PtdIns products PtdIns4*P* and PtdIns(4,5)P_2 [10]. The addition of PITP to unstimulated erythrocyte ghosts also elevated PtdIns4*P* and PtdIns(4,5)P_2 levels [11]. Furthermore, it has long been known that the total phospholipid transfer between liver organelles *in vitro* was diminished by chronic treatment with ethanol *in vivo* [12].

PITPs are cytosolic proteins originally identified by their ability to induce PtdIns transfer (reviewed in [13]). They are ubiquitous in mammalian cells. Mammalian PITP has critical roles in cellular growth [14], in polyphosphoinositide signalling [9,13], in constitutive and regulated vesicular trafficking among intracellular organelles [15,16] and in secretory trafficking pathways [17]. Its activity is strongly dependent on the physicochemical status of the membrane, the structure and charge of polar head groups, acyl-chain composition and acyl-chain order or fluidity [18]. Kinetic models supported a Ping Pong Bi Bi mechanism of lipid transfer for PITP [19,20]. Immediately downstream in distinct pathways are kinases (phosphoinositide 3-kinase and phosphoinositide 4-kinase) whose activities also are strongly dependent on lipid structure [21].

The central location of PITP in these manifestations of exposure to ethanol and the known sensitivity of PITP activity to membrane structure and order led us to investigate whether the

Abbreviations used: LUV, large unilamellar vesicle; PITP, phosphatidylinositol transfer protein; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; SUV, small unilamellar vesicle.

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activity of PITP was altered by ethanol. In the present study, the activity of recombinant mouse PITP-α was assayed *in vitro* by monitoring the transfer of radiolabelled PtdIns from rat hepatic microsomes to unilamellar vesicles in the presence of ethanol. Chloroform was examined to gauge whether the response was specific to ethanol or whether it might be shared by other membrane-fluidizing agents. Thermally pretreated microsomes and protease-treated microsomes were examined to assess the role of membrane proteins in PtdIns transfer. Several sizes of vesicle were employed to assess the effect of membrane curvature on transfer and the effect of ethanol. To evaluate the action of ethanol mechanistically, the donor and acceptor membrane concentrations were varied and the initial transfer rates were evaluated by using non-linear kinetic analyses [19].

MATERIALS AND METHODS

Materials

Cholesteryl [1-^{14}C]oleate and *myo*-[2-^{3}H]inositol were purchased from American Radiolabeled Chemicals (St Louis, MO, U.S.A.). 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) and phosphatidylinositol sodium salt from bovine liver were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Proteinase K (*Tritirachium album*, 10 units/mg) was obtained from Sigma (St. Louis, MO, U.S.A.) and used as supplied. All other chemicals were purchased from Sigma or Fisher Scientific (Pittsburgh, PA, U.S.A.). Recombinant mouse PITP-α was prepared from inclusion bodies after expression of the cDNA in *Escherichia coli* [22] and was stored at -80 °C before use.

Preparation of donor microsomes and acceptor liposomes

Microsomes were isolated from rat liver by the method of Helmkamp et al. [23]. Microsomal PtdIns was radiolabelled by head-group exchange of inositol by using *myo*-[2-^{3}H]inositol, as described previously [23,24]. Heat-treated microsomes were incubated in boiling water for 5 min under argon [25].

Protease treatment of microsomes (4.4 mg of protein/ml) was done with proteinase K (1 mg/ml) for 20 min at 37 °C. The reaction was stopped with 5 mM PMSF [added in a methanol vehicle, 1 % (v/v) final concentration]; 5 vol. of SET buffer (0.25 M sucrose/1 mM EDTA/10 mM Tris/HCl, adjusted to pH 7.4 with HCl) was added and the membranes were centrifuged at 100000 ***g*** for 1.5 h. The pellet was resuspended and washed twice in 6.25 vol. of SET buffer, and finally resuspended in the original volume of SET buffer and stored at -20 °C. The microsomal protein content was decreased by 42 %.

Lipids were dried to a thin film under a stream of N_2. The lipid film was evacuated overnight [to less than 5 mtorr (667 mPa)]. The dried lipid film was hydrated at 4 °C with SET buffer. Small unilamellar vesicles (SUVs) were obtained by sonication at 4 °C for 15 min under N_2 with a W-225 probe-type sonicator (Heat System, Farmingdale, NY, U.S.A.) at a continuous power of 40 W. The sonicated solutions were centrifuged for 10 min at 3000 ***g*** to remove titanium particles and any multilamellar vesicles. Large unilamellar vesicles (LUVs) were prepared with a freeze–thaw (five cycles) and extrusion protocol [26] that employed a lipid extruder (Avestin, Ottawa, Ontario, Canada) at ambient temperature. The LUVs were extruded by using five passes through two stacked polycarbonate filters of 0.1 or 0.2 μm pore size; these were designated LUV_{100} and LUV_{200} respectively.

Assay of phosphatidylinositol transfer

PITP transfer activity was assayed by established methods [23]. In brief, the transfer of radiolabelled [^{3}H]PtdIns from microsomes to liposomes composed of POPC and bovine liver PtdIns (98:2 molar ratio) and a trace (less than 0.01 mol %) of cholesteryl [1-^{14}C]oleate was monitored. An aliquot of PITP was added to mixtures of the donor microsomes and acceptor liposomes in SET buffer for a total volume of 250 μl. The reaction was incubated at 37 °C for 5 min unless specified otherwise. After the incubation, the samples were immersed in ice and 50 μl of acetate buffer [0.2 M sodium acetate/0.25 M sucrose (pH 5), 4 °C] was added immediately and mixed to aggregate the microsomal particles. After mixing, the samples were centrifuged at 15000 ***g*** for 20 min at 4 °C to pellet the microsomes. Samples (200 μl) of the supernatant were transferred to scintillation vials containing 0.9 ml of water and 9 ml of Bio-Safe II scintillation cocktail (Research Products International Corp., Mount Prospect, IL, U.S.A.) and analysed for ^{3}H and ^{14}C radioactivities. The radiolabels were counted in a Packard Tri-Carb model 1900CA liquid-scintillation analyser (Packard Instrument Co., Downers Grove, IL, U.S.A.) equipped with a ^{133}Ba external γ-ray source, with the use of a dual-window analysis (0–9.1 and 9.1–156 keV). Typical counting times were 5 min.

Loss of liposomes from the supernatant due to co-precipitation with the microsomal fraction (less than 20 %) was quantified by measuring the activity of cholesteryl [1-^{14}C]oleate. All calculated transfer rates accounted for loss from the liposomal fraction. Control experiments were performed without PITP to quantify spontaneous transfer. The background from spontaneous transfer was subtracted to yield PITP-mediated transfer rates. At the 5 min time point, spontaneous transfer rates were as follows (expressed as percentages of the total outer-leaflet microsomal [^{3}H]PtdIns; $n \geqslant 3$; mean $\pm$ S.D.): microsomes $\rightarrow$ SUV, 1.47 ± 0.25 %; thermally pretreated microsomes $\rightarrow$ SUV, 1.77 ± 0.35 %; microsomes $\rightarrow$ LUV_{100}, 1.13 ± 0.06 %; microsomes $\rightarrow$ LUV_{200}, 0.95 ± 0.20 %. Ethanol did not significantly alter these spontaneous transfer rates.

Kinetic treatment

The initial rate (V_0) at which PITP catalyses the PtdIns exchange between membranes was expressed by the following theoretical rate equation proposed by Van den Besselaar et al. [19] for the shuttle or Ping Pong Bi Bi mechanism relevant to PITP:

$$V_0 = \frac{k_1[\text{PtdIns}]_M \, k_2[\text{PtdIns}]_L [\text{PITP}]}{(k_1[\text{PtdIns}]_M + k_2[\text{PtdIns}]_L)(1 + K_1[\text{PtdIns}]_M + K_2[\text{PtdIns}]_L)} \quad (1)$$

where k_1 and k_2 are rate constants of association of PITP to microsomes and to liposomes respectively, and K_1 and K_2 are the binding constants of PITP to microsomes and to liposomes respectively. Our application focused on PtdIns transfer and neglected the lower phosphatidylcholine transfer capabilities of PITP. Consequently the rates are meant to be descriptive from a mechanistic standpoint, with the differences being more significant than absolute values. V_0 is dependent on the amounts of PtdIns, $[\text{PtdIns}]_M$ and $[\text{PtdIns}]_L$, present in the outer leaflet of the microsomal and liposomal membranes respectively [19]. The initial $[\text{PtdIns}]_M$ and $[\text{PtdIns}]_L$ were determined as follows. Rat hepatic microsomal membranes contain 8.5 mol % PtdIns of the total phospholipid [6]. The concentration of phospholipids in the microsome was 0.374 mg/mg of protein [27]. In microsomal membranes, PtdIns is distributed symmetrically [28]; the outer leaflet therefore contained half of the microsomal PtdIns. In curved liposomes the leaflets are not equally populated, so that

packing and mean headgroup areas vary with liposome size. In sonicated vesicles, approx. 70% of the phospholipids are in the outer leaflet [29]. The transbilayer distribution of phospholipids in SUVs depends on packing and electrostatic factors [30], resulting in a PtdIns distribution that closely resembles the inside-to-outside ratio of phosphatidylcholine [31]. The units of the fitted parameters are as follows. The association rate constant k_1 is expressed as μmol of PtdIns transferred/μmol of PtdIns in the microsomal outer leaflet per μM PITP. The association rate constant k_2 is expressed similarly, with the liposomal outer leaflet PtdIns concentration replacing that of the microsomes. Both expressions reduce to units of $\mu M^{-1} \cdot min^{-1}$. The dissociation rate constants k_{-1} and k_{-2} are expressed in units of min^{-1}. The initial velocities are expressed in units of μmol of PtdIns transferred/min per μmol of PITP.

RESULTS

Effects of ethanol on PITP activity

The dependence of the initial PtdIns transfer velocity on PITP concentration is shown in Figure 1(A) for PtdIns transfer between donor microsomes and acceptor SUVs at 37 °C. The initial transfer rate (V_0) is expressed as the percentage of the total outer-leaflet microsomal [^{3}H]PtdIns pool that was transferred to the acceptor liposomes. The PtdIns transfer rates were taken from the 5 min time point, which was well within the linear range of transfer, as shown in the time course (inset) for 0.017 μM PITP. Background transfer levels in the absence of PITP were small for this microsome/SUV assay and also for the microsome/LUV assay discussed below, as summarized in the Materials and methods section.

The presence of ethanol accelerated the PtdIns transfer mediated by PITP as shown in Figure 1(B) for the 0.017 μM PITP situation in Figure 1(A). The inset shows the time course of the ethanol enhancement, which confirmed that the 5 min point was also within the linear regime when ethanol was present. To represent more accurately the consistency of the ethanol effect, the ethanol enhancement was plotted as the increase in the rate of PITP-mediated PtdIns transfer caused by the presence of ethanol. Shown on the right-hand axis is the ethanol-induced increase in PITP activity. Transfer in the absence of PITP was not altered by ethanol (results not shown). The ethanol concentrations examined comprise the pharmacologically relevant range for rats; the range for humans is half as much [32]. The ethanol concentrations used here were well below those (more than 7 M) known to compromise liposomal integrity [33].

To assess whether the enhancement was specific to ethanol or a general response to organic solvents (i.e. a common property of anaesthetics or membrane perturbants), the effect of chloroform on PtdIns transfer is shown in Figure 1(C). Chloroform accelerated PtdIns transfer, but at much lower concentrations than was observed for ethanol. The chloroform and ethanol concentration axes shown in Figure 1(C) were matched by normalization to the respective octanol/water partition coefficients (P_{oct}) [34]. The total chloroform concentrations are shown. Aqueous concentrations were estimated to be approx. 5% less. For reference to a common biological endpoint, the arrows reflect the respective narcotic concentrations in amphibians [35]. Plotted as normalized concentrations, their efficacies are similar. Similar efficacies at concentrations normalized to P_{oct} suggest a hydrophobic mechanism of action [34].

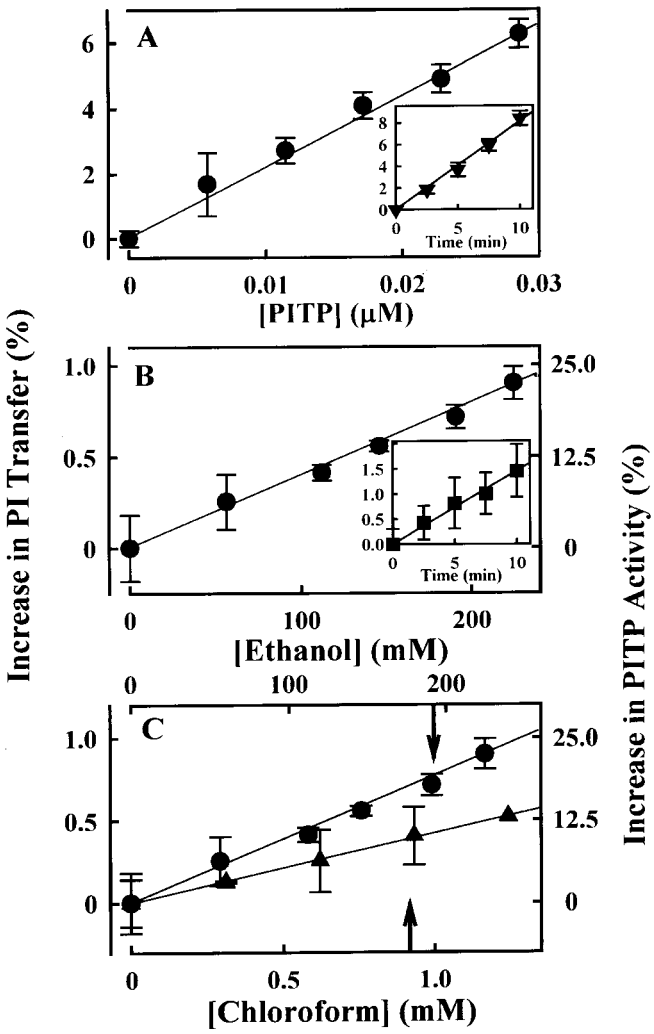

Figure 1 Activity of PITP and the effects of chloroform and ethanol

The ability of PITP to transfer PtdIns *in vitro* from donor microsomes (80 μg/ml protein) to acceptor liposomes (SUVs; 0.4 mg/ml) and the ability of ethanol and chloroform to accelerate PtdIns transfer are shown. (**A**) The PITP dose–response curve for PITP-mediated PtdIns transfer after a 5 min incubation at 37 °C is shown ($n = 4$–6). The inset shows the time course of increases in PtdIns transfer for 0.017 μM PITP ($n = 6$). (**B**) The enhancement of PITP-mediated (0.017 μM PITP) PtdIns transfer by ethanol after a 5 min incubation at 37 °C is presented as the increase in transfer caused by the presence of ethanol ($n = 3$–16). The inset shows the time course of increases in PtdIns transfer caused by ethanol (225 mM) for 0.017 μM PITP ($n = 6$). (**C**) Comparison of the enhancement of PITP-mediated PtdIns transfer by ethanol (●, $n = 3$–16) and by chloroform (▲, $n = 3$, except at 1.24 mM, for which $n = 1$) at 0.017 μM PITP. The right-hand axes in (**B**) and (**C**) show the percentage increase in the PITP activity caused by ethanol or chloroform. The concentrations of chloroform and ethanol were matched using their respective octanol/water partition coefficients [34]. The arrows represent the reported biological endpoint for narcosis in amphibians [35]. The data were fitted by linear regression; error bars show S.D. Abbreviation: PI, PtdIns.

Influence of microsomal membrane proteins on PITP activity

To address whether microsomal proteins participated in the transfer process or influenced the sensitivity to ethanol, the assay was conducted with microsomes that had been briefly incubated in boiling water (5 min) before the assay or had been subjected to protease pretreatment. The thermal treatment had previously been shown to abolish the ability of microsomes to bind ethanol saturably [25]. The protease treatment decreased the microsomal

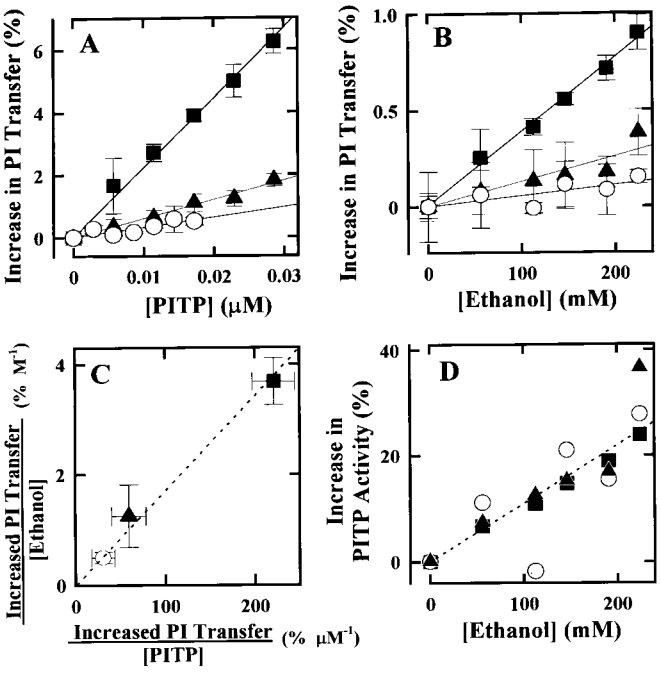

Figure 2 Effect of membrane curvature on PtdIns transfer and its enhancement by ethanol

(**A**) The increase in PITP-mediated PtdIns transfer as a function of PITP concentration is shown for highly curved sonicated vesicles (■) and for more planar extruded vesicles LUV_{100} (▲) and LUV_{200} (○). (**B**) The effect of ethanol on PtdIns transfer for the liposomes shown in (**A**) is presented as the increase in transfer caused by the presence of ethanol. (**C**) The slope from (**A**) plotted against the slope from (**B**). (**D**) The increase in PITP activity induced by ethanol for each vesicle size. All conditions were as described in the legend to Figure 1 (sonicated vesicles, $n = 3$–16; LUV_{100} and LUV_{200}, $n = 3$; error bars show S.D.). Abbreviation: PI, PtdIns.

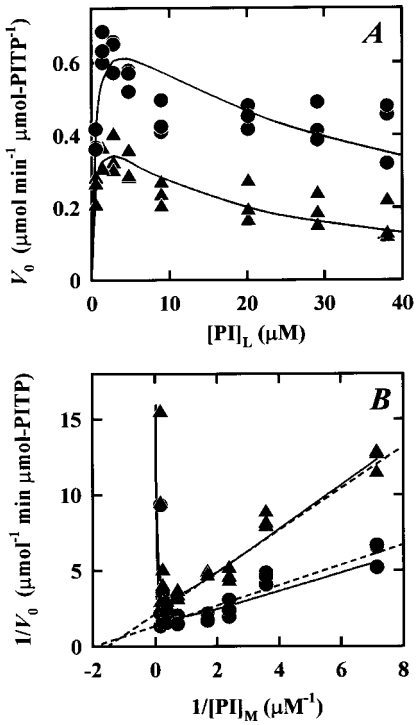

Figure 3 Kinetic analysis of PtdIns transfer

The initial velocities of PtdIns transfer were obtained for a variety of microsome and sonicated liposome (SUV) concentrations and fitted to the kinetic model of eqn. (1) with the parameters shown in Table 1. Shown are two representative slices of these data and the corresponding fits in the absence (▲) and the presence (●) of 225 mM ethanol. (**A**) The slice shown demonstrates the dependence of the initial transfer rate on the liposome concentration at constant microsome concentration (1.49 μM outer-leaflet microsomal PtdIns). (**B**) A second slice through the data set is presented in double-reciprocal format for constant liposome concentration (0.952 μM outer-leaflet liposomal PtdIns) and various microsome concentrations. Solid lines represent kinetic fits from eqn. (1); dotted lines represent regression analyses of the linear regime of the data sets. In both panels the liposome and microsome concentrations were expressed in terms of the concentration of PtdIns on the outer leaflet of the liposome or microsome (see the text). Abbreviation: PI, PtdIns.

protein content by 42%. Neither thermal pretreatment nor protease pretreatment of the donor microsomes affected the PITP-mediated PtdIns transfer or its ethanol enhancement (results not shown). This result was consistent with the microsomal proteins neither being involved in the transfer process nor being the source of the hydrophobic site that was responsible for the response to ethanol.

Effects of liposomal size/curvature on PITP activity and its ethanol enhancement

Figure 2 presents the effects of liposomal size on PtdIns transfer in the absence and the presence of ethanol. PITP-mediated PtdIns transfer was sharply diminished with the increased size of the acceptor vesicles (Figure 2A). The most planar acceptor membranes exhibited the slowest rates of transfer; conversely, the most curved membranes exhibited the fastest rates of transfer. The relative rates were as follows: SUV, 7.1; LUV_{100}, 1.9; LUV_{200}, 1.0. The dose response to ethanol mirrored that of PITP concentration, as shown in Figure 2(B). The ethanol-induced increase in PITP-mediated PtdIns transfer was least for the most planar membranes and greatest for the most curved membranes. However, when the decreased activity of PITP in the larger vesicles was accounted for, the enhancement of PITP activity by ethanol was independent of vesicle size. The slopes in Figures 2(A) and 2(B) are plotted in Figure 2(C) to indicate that changes in the degree of curvature exerted similar effects on the dose–response curves of both PITP and ethanol. Similarly, when the influence of ethanol was expressed in terms of its ability to enhance the PITP activity, no dependence on curvature could be discerned (Figure 2D). The effect of ethanol was applicable to membranes of all degrees of expressed curvature.

Kinetic analysis of the ethanol enhancement

A kinetic analysis of PtdIns transfer was undertaken to provide a mechanistic description of the enhancement by ethanol. Initial transfer velocities were obtained as a function of donor and acceptor membrane concentrations. A three-dimensional non-linear regression analysis of the initial velocities to the theoretical rate equation [eqn (1)] was performed. A representative slice through the data set is shown in Figure 3(A) for a constant microsome concentration and various liposome concentrations in the presence and the absence of ethanol. The sharp enhancement by ethanol is evident, and the fit to eqn. (1) was reasonable.

The fitted parameters (k_1, k_2, K_1 and K_2) are summarized in Table 1. Parameters k_{-1} and k_{-2}, the dissociation rate constants

Table 1 Effects of ethanol on kinetic parameters

k_1, k_2, K_1 and K_2 were obtained from non-linear regression analysis based on 63 values (n) for outer-leaflet concentrations $[PtdIns]_M = 0.14–5.9\ \mu M$ and $[PtdIns]_L = 0.20–38\ \mu M$, at $0.034\ \mu M$ [PITP]. k_{-1} and k_{-2} were calculated from $k_{-1} = k_1/K_1$ and $k_{-2} = k_2/K_2$. r^2 is the correlation coefficient of the regression analysis. Results are presented as means ± S.E.M.

Ethanol (mM)	k_1 ($\mu M^{-1} \cdot min^{-1}$)	k_{-1} (min^{-1})	k_2 ($\mu M^{-1} \cdot min^{-1}$)	k_{-2} (min^{-1})	K_1 (μM^{-1})	K_2 (μM^{-1})	n	r^2
0	23 ± 3	23	78 ± 28	487	1.0 ± 0.3	0.16 ± 0.04	63	0.81
225	52 ± 11	32	200 ± 81	1834	1.7 ± 0.6	0.11 ± 0.03	63	0.74

of PITP from microsomes or SUVs respectively, were calculated from the following relations: $k_{-1} = k_1/K_1$ and $k_{-2} = k_2/K_2$ [19]. Ethanol (225 mM) approximately doubled the association and dissociation rate constants, indicating that it lowered the activation barrier to the PITP-mediated insertion and desorption of PtdIns. In contrast, the binding of PITP to the membranes was largely unaffected.

A second slice through the data set is presented in Figure 3(B) in the double-reciprocal format occasionally used to assess the activities of phospholipid transfer proteins [20]. In the slice shown, the microsome concentration was varied at a constant liposome concentration. The solid lines represent the fits from eqn. (1) that were shown in Figure 3(A) and compiled in Table 1. The upturn at high membrane concentrations is characteristic of phospholipid transfer proteins and was reproduced by the kinetic model. This effect arises largely from a diminution of the aqueous PITP concentration owing to binding at the microsomal membrane surface; an analogous decrease in the initial transfer velocity at high liposome concentrations is apparent in Figure 3(A). The dotted lines show a regression analysis of the linear portion of the curve. K_m was not significantly altered (0.66 ± 0.10 to 0.55 ± 0.15 μM for outer-leaflet microsomal PtdIns in the absence and the presence of ethanol respectively). Ethanol enhanced V_{max} (0.46 ± 0.05 to 0.80 ± 0.15 μmol/min per μmol of PITP in the absence and the presence of ethanol respectively).

DISCUSSION

Ethanol and PITP

The activity of PITP is very sensitive to the physical character of membranes. Acyl-chain order and surface charge greatly influence the activity of PITP [18], with PtdIns transfer being enhanced at more disordered or fluidized membranes [18]. Here we have illustrated the steep functional dependence on lipid structure by showing that the activity of PITP is strongly dependent on the expressed curvature of the acceptor vesicles. Steep functional dependences on expressed curvature have previously been reported for phosphatidylcholine transfer protein [36,37], phosphoinositide 4-kinase and phosphoinositide 3-kinase [21]. Ethanol enhanced the activity of PITP by increasing the PITP-mediated flux of PtdIns at the membrane surface. This ability to enhance PITP activity was not unique to ethanol: chloroform also enhanced PITP activity. Chloroform shares ethanol's ability to disorder or fluidize membranes. Their abilities to disorder membranes are correlated with their respective octanol/water partition coefficients [38]. Similarly, their abilities to enhance PITP transfer are correlated with their octanol/water partition coefficients. This result suggests that an enhancement of PITP activity could be a general consequence shared by membrane-fluidizing agents. However, membrane fluidity is only one of many biochemical processes involving hydrophobic sites that are correlated with octanol/water partitioning [39]. To investigate the role of microsomal proteins in the activity of PITP or its ethanol enhancement, transfer from thermally pretreated microsomes was monitored. We have shown previously that brief thermal pretreatment abolished the saturable binding of ethanol in microsomes [25]. Thermal pretreatment of the donor microsomes did not alter the activity of PITP and did not alter the enhancement by ethanol. Similar results were obtained with protease-treated microsomes. It is therefore unlikely that microsomal proteins or hydrophobic regions within them have a direct role in PITP transfer.

The activity of PITP depends inversely on the size of the liposomes. Smaller vesicles exhibit greater local convex curvature, greater headgroup areas and smaller cohesive forces in the outer leaflet in comparison with larger, more planar vesicles [40]. Similarly, ethanol and chloroform are membrane perturbants that fluidize membranes, increase headgroup areas and decrease cohesive forces between membrane lipids. PITP is very sensitive to the cohesive forces in membranes: a correlation between cohesive forces and phospholipid extraction has been postulated [41]. Ethanol enhanced the spontaneous desorption of fluorescent phospholipid analogues from lipid bilayers owing to its ability to decrease the cohesive forces between phospholipids in membranes [42]. The kinetic analysis supported this reasoning by showing that ethanol increased the successful insertion/desorption rates without significantly affecting PITP binding. The potential importance of the cohesive forces or membrane surface tension on PITP activity can be understood from the phospholipid exchange mechanism formulated from the recent crystal structure of the non-homologous yeast PITP [43]. The yeast PITP contained an unusual surface helix that was proposed to act like a swinging door, swinging into the membrane to sweep open a cavity and deposit the incoming phospholipid, then swinging back to abstract the outgoing phospholipid. The model was so disruptive to the membrane phospholipids that the authors termed it the 'bulldozer model'. Thus phospholipid exchange might be influenced by lateral cohesive forces between the membrane lipids that, in turn, were altered by curvature, chloroform or ethanol.

A dynamic viewpoint gives rise to a subtly different, but complementary, physical interpretation of ethanol's actions. Alcohols and other non-polar solutes are known to induce fluctuations in the shape of membranes by locally reducing the bending modulus (membrane stiffness) [44,45]. Consequently the alcohol-containing membranes are more malleable and more prone to local deformations, which would reduce the energy barriers to transfer.

The reasoning for a lipid-mediated pathway of ethanol action is based on a comparison of the effects of membrane structure on PITP activity and the known effects of ethanol on membrane structure, with the locus being the cohesiveness of the membrane lipids. An alternative explanation is that ethanol or chloroform binds to the hydrophobic acyl-chain-binding sites of PITP to enhance transfer. If this were true, we would expect

transfer to be diminished, not enhanced. When alcohols and anaesthetics bind to hydrophobic clefts meant to dock with hydrophobic substrates, they interfere with or weaken substrate binding [46]. However, it is the competition between the association energy of the PtdIns–PITP complex and the association energy of the bilayer lipids (cohesiveness) that determines the transfer rate (for constant electrostatic factors) [41]. When the binding strength is much greater than the lipid cohesiveness, the surface flux is high. It is thought that the extraction/desorption step of the transfer process is rate-limiting, owing to the difficulty of overcoming the cohesive forces in the membrane, as is necessary for lipid extraction. If ethanol were to act by binding to the hydrophobic cleft, it would weaken the strength of the PtdIns–PITP complex and decrease the speed of transfer.

Implications for ethanol action

Historically, explanations for the common actions of organic solvents (anaesthetics) have fluctuated between protein, lipid and interface theories. More recently, opinion has shifted from lipid theories to protein theories as evidence has accumulated that clearly implicate hydrophobic regions of proteins as common targets of alcohols and anaesthetics [25,47]. This has led some to question whether any of the functional effects of alcohols and anaesthetics are mediated by membrane lipids [47]. Persuasive evidence for lipid-mediated actions of ethanol has been presented but is limited [48–50]. The sensitivity to ethanol described here for PITP rivals or exceeds that of any previously described lipid-mediated action of ethanol. Ethanol is best viewed as a very promiscuous molecule that acts on the hydrophobic regions of all biomaterials.

PITP might be a prototypical example for proteins that are sensitive to ethanol's effects on bulk lipids. Foremost, its function is exquisitely sensitive to membrane structure. By comparison, its sensitivity to ethanol is modest, being consistent with the modest lipid perturbations exerted by pharmacological concentrations of alcohols and anaesthetics [4]. A steep functional dependence on lipid structure and a comparatively modest functional dependence on alcohol are likely to be common features of proteins influenced by ethanol's effects on membrane lipids. Thus a strong sensitivity of proteins to lipid structure is likely to be a prerequisite for mechanisms of ethanol action mediated by bulk lipids.

Membrane curvature and PITP

It is a puzzle why PITP is critical to many biochemical processes. Membranous PtdIns stores are known not to be depleted significantly in processes that require PITP [9,11]. PITP seems to deliver PtdIns to metabolically active sites in the membrane, rather than passively maintaining the membrane's overall PtdIns content. The marked sensitivity of PITP to membrane structure provides a plausible mechanism for the ability of PITP to direct the delivery of PtdIns to metabolically active membrane domains. Transfer to membrane regions of convex curvature or low lipid surface density, as shown above, is markedly enhanced in comparison with transfer to planar regions. Because phosphoinositide 3-kinase and phosphoinositide 4-kinase also are much more active at convex surfaces [21], the potential exists for exponential increases in the production of PtdIns3*P* and PtdIns4*P* at curved membrane regions.

We thank Jan B. Hoek and George M. Helmkamp Jr for valuable discussions. This work was supported by National Institutes of Health grants AA00163, AA07186 and AA07463, and by the Alcoholic Beverage Medical Research Foundation.

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Received 2 November 1999/14 March 2000; accepted 11 April 2000