Biochem. J. (1988) 254, 147–153 (Printed in Great Britain)

Alcohol-induced stimulation of phospholipase C in human platelets requires G-protein activation

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In previous studies we have demonstrated that ethanol activates hormone-sensitive phospholipase C in intact human platelets, resulting in the mobilization of intracellular Ca^{2+} and platelet shape change. The present study aims to localize further this effect of ethanol by examining its interaction with the regulation of phospholipase C in a permeabilized cell system. In platelets permeabilized with a minimal concentration (18 μg/ml) of saponin, ethanol by itself did not activate phospholipase C. However, ethanol potentiated the activation of phospholipase C in response to the non-hydrolysable GTP analogue GTP[S] (guanosine 5'-[γ-thio]triphosphate), an effect similar to that observed with thrombin. Ethanol also potentiated the response to fluoride, which acts directly on G-proteins. Other short-chain alcohols also stimulated phospholipase C in a synergistic manner with GTP[S]. The ability of specific alcohols to stimulate phospholipase C was directly related to their respective lipid-solubilities, as determined by their partition coefficients. Moreover, the potencies of each alcohol correlated with their ability to elicit Ca^{2+} mobilization and shape change in intact platelets. These effects of ethanol were eliminated by a disruption of receptor–phospholipase C coupling induced by the addition of higher concentrations of saponin. These data indicate that the activation of phospholipase C by ethanol may occur by affecting protein–protein interactions in the signal-transduction complex involving GTP-binding regulatory proteins.

INTRODUCTION

In a series of studies (Hoek *et al.*, 1987,1988; Rubin & Hoek, 1988; Rubin *et al.*, 1987, 1988) we investigated the effects of ethanol on the phosphoinositide-linked second-messenger system. Addition of ethanol (25–500 mM) to suspensions of isolated rat hepatocytes (Hoek *et al.*, 1987; Rubin & Hoek, 1988) and human platelets (Rubin *et al.*, 1988) was found to induce an activation of phosphoinositide-specific phospholipase C, with kinetic characteristics resembling those of the agonist-stimulated process. The resultant increase in intracellular inositol 1,4,5-trisphosphate caused the release of intracellular Ca^{2+} from hormone-sensitive stores, leading to an increase in cytosolic Ca^{2+} and a subsequent activation of glycogen phosphorylase in hepatocytes (Hoek *et al.*, 1987) and the phosphorylation of myosin light chain in human platelets (Rubin *et al.*, 1988). Ethanol also stimulated the phosphorylation of a 40–47 kDa protein in platelets, a known substrate of protein kinase C, presumably in response to phospholipase C-generated diacylglycerol. The coordinated action of these intracellular signals in human platelets induced a rapid shape change, but did not cause aggregation or secretion (Rubin *et al.*, 1988). Thus ethanol mimicked some, but not all, of the effects of receptor-mediated agonists acting through phospholipase C.

The mechanism by which ethanol stimulates phospholipase C is not known. Studies on hepatocytes suggested that the effect was not specific for ethanol; a variety of other hydrophobic solvents and general anaesthetics induced a similar response, including some that have little structural resemblance to short-chain alcohols (Hoek *et al.*, 1987). It is therefore unlikely that the effect relies on the specific interaction of ethanol with a recognition site on a receptor molecule. Yet there is evidence that ethanol interacts at an early stage with the control of phospholipase C activity. Specifically, the ethanol-induced activation of phospholipase C was found to be inhibited by pretreatment of hepatocytes with phorbol esters that activate protein kinase C (Hoek *et al.*, 1988). Phospholipase C activation by certain hormones is also inhibited by phorbol ester pretreatment (Lynch *et al.*, 1985; Cooper *et al.*, 1985). The target of protein kinase C may be either a receptor or a G-protein which is presumed to be involved in regulating the interaction between receptors and phospholipase C in the plasma membrane (Lynch *et al.*, 1985; Cooper *et al.*, 1985).

Attempts to demonstrate directly the ethanol-induced activation of phospholipase C in plasma-membrane preparations or in permeabilized cells have so far been unsuccessful (Rubin *et al.*, 1987). Although phospholipase C activity in digitonin-permeabilized hepatocytes could be stimulated by the non-hydrolysable GTP analogue GTP[S], indicating the preservation of G-protein control of the enzyme, ethanol had no effect on phospholipase C activity at any concentration of GTP[S]. In this system, however, receptor-mediated control of phospholipase C was lost, suggesting the possibility that

Abbreviations used: GTP[S], guanosine 5'-[γ-thio]triphosphate; p[NH]ppG, guanyl-5'-yl imidodiphosphate; GDP[S], guanosine 5'-[β-thio]diphosphate.

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the action of ethanol requires the integrity of the receptor–G-protein–phospholipase C complex.

In this paper we have used saponin-permeabilized platelets to analyse further the site of action of ethanol and other short-chain alkanols, since other studies (Authi *et al.*, 1986; Brass *et al.*, 1986) have shown that a significant degree of receptor-controlled phospholipase C activity is more readily retained in this system. The results provide direct evidence that ethanol can interact with the cell membrane to stimulate phosphoinositide-specific phospholipase C. Stimulation depends on the presence of GTP[S] or other agents that activate G-proteins. Other short-chain alcohols have similar effects, with potencies that are proportional to their ability to interact with phospholipid membranes. These effects are abolished in parallel with a disruption of receptor-mediated phospholipase C coupling.

EXPERIMENTAL

Materials

myo-[2-^{3}H]Inositol (15 Ci/mmol in 95% ethanol) was purchased from American Radiolabeled Chemicals. Before use, the ethanol was removed by evaporation in a Rotovac evaporator. The radiochemical was then resuspended in Hepes buffer and treated with a small amount of Dowex AG 1-X8 resin (Bio-Rad) to remove charged impurities. GTP, GTP[S], p[NH]ppG and GDP[S] were obtained from Boehringer Mannheim. Fura2 penta-acetoxymethyl ester was purchased from Calbiochem, [^{32}P]ATP from Amersham, and saponin from Sigma. All other chemicals and biochemicals, of the highest purity commercially available, were purchased from Sigma or Fisher Scientific.

Platelet preparation and labelling with [^{3}H]inositol

The procedure for labelling platelets with [^{3}H]inositol was adapted from the methods described by Lapetina and co-workers (Lapetina, 1986; Siess & Lapetina, 1986). Blood (60 ml) was obtained from healthy human volunteers who had not received medication in the previous 10 days. Blood was anticoagulated with 0.38% trisodium citrate. Platelet-rich plasma was obtained by centrifugation of whole blood at 200 *g* for 15 min at room temperature. EDTA (5 mM) and prostacyclin (1 μM) were added to the platelet-rich plasma, which was then centrifuged at 800 *g* for 20 min at room temperature. The platelet pellet was resuspended in 1 ml of Hepes buffer (145 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 10 mM-Hepes, pH 7.4 at 37 °C, plus 10 mM-glucose) containing 1 mM-EGTA, 10 nM-prostacyclin, 10 μM-indomethacin and 0.3 mCi of *myo*-[2-^{3}H]inositol, and incubated in a shaking water bath at 37 °C for 3 h. After labelling, the platelets were washed in 35 ml of acid/citrate/dextrose-treated Hepes buffer (pH 6.4) containing 1 μM-prostacyclin. The pellet was resuspended in 'incubation' buffer, containing 137 mM-NaCl, 2.7 mM-KCl, 1 mM-$MgCl_2$, 3.3 mM-NaH_2PO_4 and 20 mM-Hepes, pH 7.4, plus 1 mM-EGTA, 10 mM-LiCl, 10 μM-indomethacin, 2 mM-phosphocreatine and 2 units of creatine kinase/ml. The final platelet concentration was adjusted to $(3–3.5) \times 10^8$/ml. The final free Ca^{2+} concentration of the incubation buffer was adjusted to 0.1 μM (verified by use of the fluorescent calcium indicator quin-2; Tsien, 1981).

Preparation of permeabilized platelets

Platelets were permeabilized with saponin (18 μg/ml) essentially as described previously by others (Brass & Joseph, 1985; Lapetina *et al.*, 1985; Authi *et al.*, 1986). Portions (0.2 ml) of [^{3}H]inositol-labelled or unlabelled platelets were pre-warmed at 37 °C in a shaking water bath for 5 min, followed by a 2 min incubation with saponin (18 μg/ml) and 1 mM-ATP. Futher incubations of the permeabilized platelets were carried out immediately after permeabilization in the standard incubation medium, with additions as indicated in the Figure legends.

Determination of [^{3}H]inositol phosphates

Phospholipase C activity was determined by measurement of total [^{3}H]inositol phosphates after incubation of the permeabilized platelets in the presence of 10 mM-Li^+. At the indicated times, 0.15 ml platelet samples were quenched in 0.56 ml of chloroform/methanol/HCl (40:20:1, by vol.). The phases were then separated by addition of 0.188 ml of 2 M-KCl/10 mM-EDTA, pH 7.0, and 0.188 ml of chloroform. After centrifugation (800 *g*), the chloroform layers were taken and the remaining aqueous phases washed with 0.375 ml of chloroform. The aqueous phases were separated, neutralized to pH 7.0 with 2 M-KOH/10 mM-Mops and diluted in 10 ml of water. Free *myo*-inositol, glycerophosphoinositides and inositol phosphates were separated by anion-exchange chromatography on 0.5 ml Dowex 1-X8 anion-exchange-resin columns (formate form; 200–400 mesh) as described previously (Thomas *et al.*, 1984). The ^{3}H content of the total inositol phosphates was determined by liquid-scintillation counting in BudgetSolve (Research Products International). Unless otherwise indicated, the results are expressed as percentages of control values and are the means of at least three experiments performed in duplicate or triplicate. Control values for total inositol phosphates in at least 20 experiments ranged from 300 to 800 c.p.m.; the variation reflected differences in the degree of incorporation of [^{3}H]inositol between preparations.

Phosphatidic acid formation

Unlabelled washed platelets were prelabelled with [^{32}P]ATP by incubation in the standard buffer (phosphocreatine and creatine kinase omitted) in the presence of saponin (18 μg/ml), ATP (1 mM) and [^{32}P]ATP (4 μCi/ml) for 2 min. Subsequently, other additions were made as indicated for different experiments and incubations were continued for 4 min. Samples were quenched in chloroform/methanol/HCl, and phases were separated as described above. [^{32}P]Phosphatidic acid was measured in the chloroform layer as described previously (Hoek *et al.*, 1987) by t.l.c. on oxalate-treated silica-gel 60 plates (EM Reagents), with a solvent system of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) (Jolles *et al.*, 1979). The ^{32}P content of phosphatidic acid was quantified by liquid-scintillation counting.

Measurement of platelet shape change

Shape change was determined in a Chronolog aggregometer at 37 °C, with stirring at 1000 rev./min.

Measurement of cytosolic free Ca^{2+} in Fura-2-loaded platelets

Platelet-rich plasma was incubated with 4 μM-Fura2 (ester) at 37 °C for 30 min. The platelets were pelleted by centrifugation (800 *g*) and resuspended in Hepes buffer at a concentration of 2×10^8/ml. Cytosolic free Ca^{2+} was determined as described by Grynkiewicz *et al.* (1985), as described previously (Rubin *et al.*, 1988).

Statistics

Statistical significance was determined by Student's *t* test.

RESULTS

Several authors (Brass & Joseph, 1985; Lapetina *et al.*, 1985; Authi *et al.*, 1986) have shown that saponin in low concentrations causes a selective permeabilization of the plasma membrane of human platelets, while preserving a significant degree of receptor-mediated phospholipase C activation. In such a system, the control of phospholipase C activity can be studied more readily, since it is possible to use non-permeant agents that interfere with normal control functions of the system, e.g. the non-hydrolysable analogues of GTP which interact with G proteins. Moreover, in permeabilized cells the activity of phospholipase C can be measured more accurately, since secondary metabolism of its products can be readily controlled and feedback-control features operative in the intact cell are not active. These features are illustrated in the experiment of Figs. 1 and 2. Platelets were permeabilized with saponin (18 μg/ml) and incubated in a medium containing LiCl (10 mM) which prevents the hydrolysis of inositol monophosphate, thereby allowing the accumulation of inositol phosphate products of phosphatidylinositol 4,5-bisphosphate degradation. ATP and an ATP-regenerating system were added to allow for the continuous resynthesis of substrate polyphosphoinositides. The free Ca^{2+} concentration was maintained at 0.1 μM and indomethacin was included to prevent feedback stimulation of phospholipase C by arachidonic acid metabolites. Under these conditions, an increase in total inositol phosphates reflects activation of phospholipase C. As shown in Fig. 1, the production of inositol phosphates was linear with time over a period of at least 20 min in the presence of GTP[S] (50 μM) and was significantly stimulated by ethanol. This time course differs markedly from that observed in intact cells (Hoek *et al.*, 1987, 1988; Rubin *et al.*, 1988).

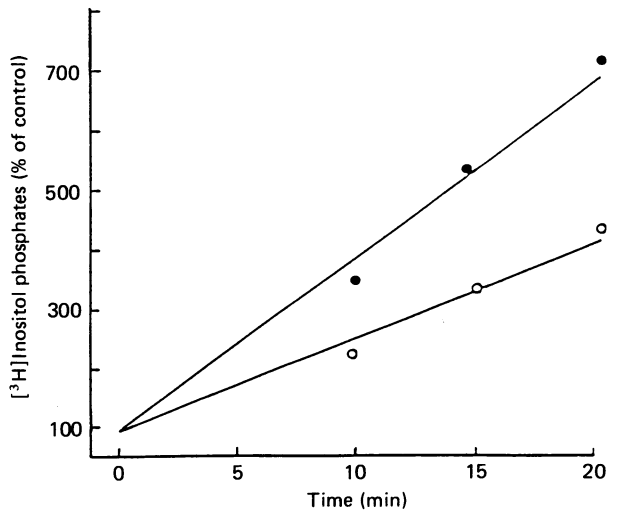

Fig. 1. Effect of ethanol on the time course of inositol phosphate formation induced by GTP[S] in permeabilized platelets

[^{3}H]Inositol-labelled platelets were permeabilized with saponin for 2 min at 37 °C before the addition of 50 μM-GTP[S] at zero time in the absence (○) or presence (●) of 300 mM-ethanol. At the indicated times, samples were obtained for analysis of total [^{3}H]inositol phosphates. The data are from one of two similar experiments performed in duplicate, and are expressed as percentages of values obtained before addition of GTP[S].

In intact platelets, the ethanol-induced increase in cytosolic Ca^{2+} reaches a peak after 1–5 s and subsides to near-basal values within 2 min (Rubin & Hoek, 1988). The reasons for the transient nature of this effect of ethanol are unknown; however, in hepatocytes feedback inhibition by protein kinase C has been implicated in a similar response (Hoek *et al.*, 1988). This apparent feedback control of phospholipase C activation appears to be lost on permeabilization. The stimulation of phospholipase C by ethanol in permeabilized platelets was not altered by addition of ethanol 10 min before addition of saponin (results not shown) indicating that a pretreatment to induce desensitization does not prevent the stimulation of phospholipase C by ethanol in permeabilized cells.

Fig. 2 shows the dependence of phospholipase C activation on the GTP[S] concentration in the presence and the absence of ethanol. In agreement with previous reports (Brass *et al.*, 1986), GTP[S] stimulated phospholipase C activity in permeabilized platelets at concentrations between 0.1 and 10 μM, with maximal stimulation occurring at approx. 10 μM-GTP[S]. Concomitant with inositol phosphate accumulation (Fig. 2*a*), GTP[S] also stimulated phosphatidic acid formation (Fig. 2*b*); the latter is presumed to be generated from diacylglycerol, the second product of phospholipase C. However, other pathways of diacylglycerol metabolism are probably also available in the permeabilized cells. In the absence of GTP[S], ethanol (300 mM) had no significant effect on either inositol phosphate formation or phosphatidic acid production. However, at GTP[S] concentrations greater than 1 μM, ethanol significantly enhanced phospholipase C activity (Fig. 2). The addition of 300 mM-ethanol resulted in a 1.76 ± 0.06-fold enhancement of phospholipase C activity in the presence of a maximal concentration of GTP[S] (50 μM) (mean ± S.E.M. of six experiments; $P < 0.01$). Ethanol had no effect on the apparent K_d of GTP[S] for stimulating phospholipase C; half-maximal stimulation was obtained at 1–5 μM-GTP[S] in both the absence and the presence of ethanol. In other experiments (results not shown), thrombin was also found to act synergistically with GTP[S] in enhancing phospholipase C activity, in agreement with the findings reported by other workers (Rock & Jackowski, 1987; Brass *et al.*, 1986).

Addition of the non-hydrolysable GTP analogue p[NH]ppG or NaF also stimulated phospholipase C in the permeabilized cells. Ethanol caused a 49 ± 8% increase in NaF-stimulated phospholipase C activity (mean ± S.E.M.; $n = 3$). In four experiments, ethanol also potentiated the response to p[NH]ppG and GTP; however, the effects were inconsistent. GDP did not

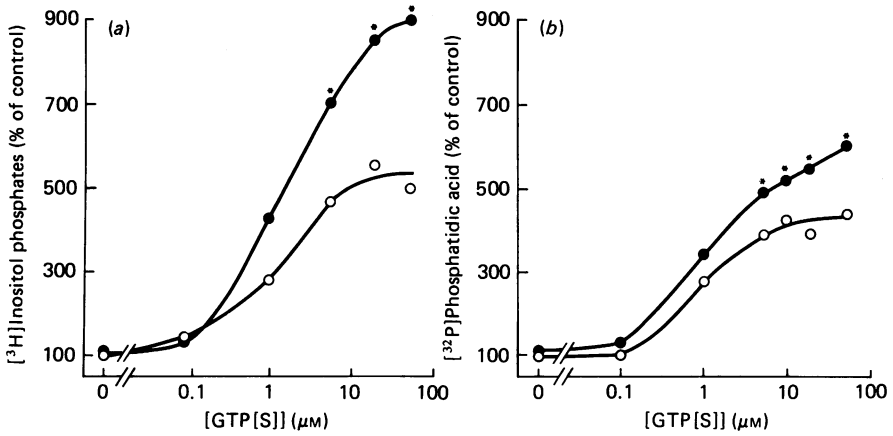

Fig. 2. Effect of ethanol on the concentration-dependence of GTP[S]-stimulated phospholipase C in permeabilized platelets

Washed platelets were permeabilized by the addition of saponin (18 μg/ml) for 2 min, before addition of the indicated concentrations of GTP[S] either with (●) or without (○) ethanol (300 mM). (*a*) [^{3}H]Inositol phosphates were measured by anion-exchange chromatography after 15 min incubation. (*b*) [^{32}P]Phosphatidic acid formation was determined by t.l.c. after 6 min. Further details of the incubation and extraction procedures were as described in the Experimental section. Each point is the mean ± S.E.M. of values obtained from three separate experiments, with individual measurements expressed as the percentage of the basal value within that experiment: $*P < 0.05$.

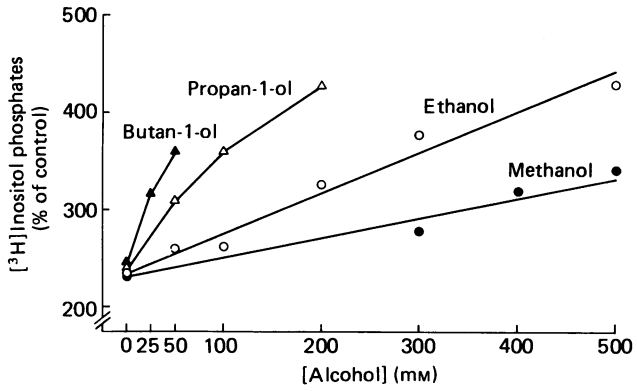

Fig. 3. Concentration-dependence of various short-chain alcohols for phospholipase C activation in saponin-treated platelets

Saponin-permeabilized platelets were incubated for 15 min with 50 μM-GTP[S] in the absence or presence of the indicated concentrations of methanol (●), ethanol (○), propan-1-ol (△) and butan-1-ol (▲). After a 15 min incubation period, [^{3}H]inositol phosphates were determined as described in the Experimental section. Each point is the mean of three separate experiments, and is expressed as the percentage of values obtained from incubations in the absence of GTP[S].

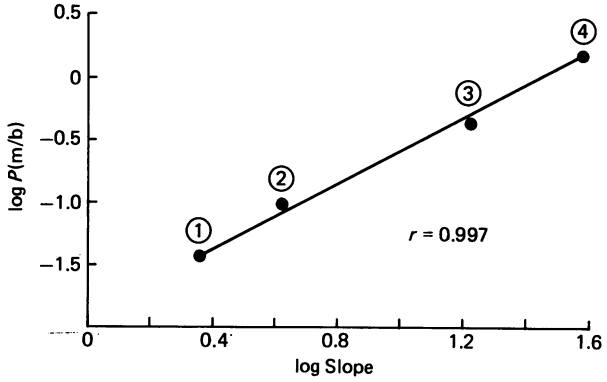

Fig. 4. Correlation of phospholipase C stimulation by short-chain alcohols with membrane/buffer partition coefficients

The potency of individual alcohols in stimulating phospholipase C is expressed as the log slope of the linear portion of the concentration-dependence curves in Fig. 3. Membrane/buffers partition coefficients, *P*(m/b), were obtained from McCreery & Hunt (1978), as determined by the octanol/water partitioning method. The numbers in the Figure represent different alcohols; methanol (1), ethanol (2), propan-1-ol (3) and butan-1-ol (4).

stimulate inositol phosphate production, and ethanol had no significant further effect with this agent. The inositol phosphate response to all agents tested was paralleled by similar increases in [^{32}P]phosphatidic acid formation (results not shown).

The potency of different concentrations of ethanol and several other short-chain alcohols in stimulating GTP[S]-activated phospholipase C is shown in Fig. 3. Methanol, ethanol, propan-1-ol and butan-1-ol all potentiated GTP[S]-dependent phospholipase C activity in a concentration-dependent manner, with methanol being the least potent and butan-1-ol the most effective. The concentration-dependence of phospholipase C stimulation was linear for ethanol and methanol up to concentrations of 500 mM; significant enhancement of GTP[S]-stimulated phospholipase C was obtained at 50 mM-ethanol and 300 mM-methanol. Concentrations of butan-1-ol and propan-1-ol greater than 25 mM and 200 mM, respectively, were inhibitory (results not shown).

Many of the effects of short-chain alcohols correlate with their membrane/buffer partition coefficients [*P*(m/b)] (Taraschi & Rubin, 1985; McCreery & Hunt, 1978). We therefore compared the potency of each alcohol in stimulating phospholipase C with their respective partition coefficients (Fig. 4). The relative potency of each alcohol was calculated from the initial slope of the concentration/response curves in Fig. 3. The log of the slope of phospholipase C activity was linearly related to

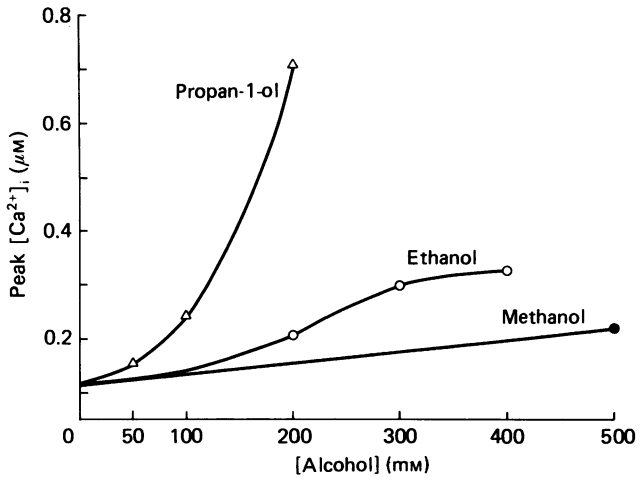

Fig. 5. Effect of alcohols on cytosolic free Ca^{2+} in Fura 2-loaded platelets

Fura2-loaded platelets were preincubated at 37 °C in Hepes buffer containing 1 mM-$CaCl_2$ for 5 min before addition of the indicated concentration of each alcohol. Fluorescence changes were measured at an emission wavelength of 510 nm and an excitation wavelength of 340 nm. Cytosolic free calcium, $[Ca^{2+}]_i$, was determined as described in the Experimental section. Each point represents the peak $[Ca^{2+}]_i$ obtained within 2–3 s.

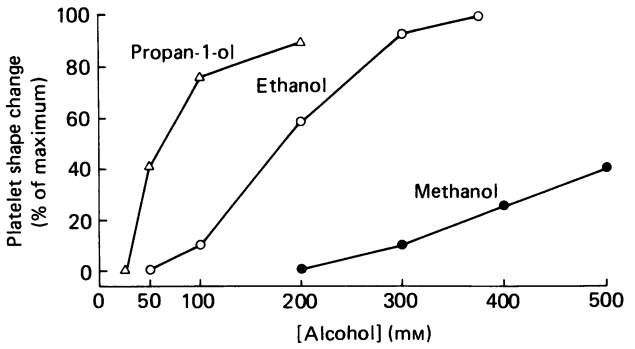

Fig. 6. Alcohol-induced shape change in intact human platelets

Platelet-rich plasma was prepared from 0.38% trisodium citrate-treated blood. Samples (0.5 ml) were stirred in aggregometer tubes in a Chronolog aggregometer at 37 °C for at least 5 min before addition of the indicated concentrations of methanol (●), ethanol (○) and propan-1-ol (△). Shape change was as indicated by a decrease in light transmission, and was maximal at 400 mM-ethanol. Each point represents the mean of three separate experiments and is expressed as the percentage of maximum shape change. Data were obtained after 2 min of alcohol exposure, at which time all responses were maximal.

the log of the partition coefficients, with a slope of approx. 1. Thus these data support our suggestion (Hoek *et al.*, 1987) that the ability of a specific alcohol to stimulate phospholipase C is directly related to its ability to interact with the membrane. A similar relationship was obtained for the ability of short-chain alcohols to cause disordering of the membrane bilayer structure, as detected by e.s.r. (see Taraschi & Rubin, 1985).

The activation of phospholipase C in intact platelets leads to the mobilization of Ca^{2+} and the stimulation of myosin light-chain phosphorylation, thereby resulting in platelet shape change (Zucker & Nachmias, 1985). The ability of various short-chain alcohols to stimulate phospholipase C was further investigated in intact platelets loaded with the Ca^{2+} indicator Fura2. The characteristics of ethanol-induced Ca^{2+} mobilization in platelets have been detailed previously (Rubin & Hoek, 1988). As shown in Fig. 5, methanol and propan-1-ol were both effective in increasing cytosolic Ca^{2+}, with propan-1-ol being 5–10-fold more effective than methanol. The kinetics of Ca^{2+} release were similar for all alcohols tested, with peak cytosolic Ca^{2+} values obtained within 1–5 s (results not shown). Fig. 6 illustrates the potencies of the various alcohols in stimulating shape change. The results correlate closely with the ability of different alcohols to activate phospholipase C in permeabilized platelets (Fig. 3) and to evoke Ca^{2+} mobilization in intact platelets (Fig. 5).

The data of Figs. 1–3 establish that ethanol can stimulate phospholipase C in permeabilized platelets, provided that GTP[S] is present. This observation differs from our previous findings in digitonin-permeabilized hepatocytes, where ethanol had no effect under similar conditions (Rubin *et al.*, 1987). The experiment of Fig. 7 suggests that this difference might be explained by the fact that activation of phospholipase C by ethanol is dependent on the presence of functional receptor-linked pathways. At the standard concentration of saponin (18 μg/ml) the activation of phospholipase C by GTP[S] was potentiated by both ethanol and thrombin. GDP[S] inhibited thrombin-induced inositol phosphate production, demonstrating that the effect of thrombin was not due to the presence of a significant fraction of non-permeabilized cells. In contrast with ethanol, thrombin caused some increase in inositol phosphate production in the absence of GTP[S] at a saponin concentration of 18 μg/ml. Ethanol is a poor stimulant when GTP alone is present. Presumably this was largely due to the presence of endogenous GTP, since GDP[S] also inhibited this activity. When increasing concentrations of saponin were used for permeabilization, there was a dramatic loss of the ability of both ethanol and thrombin to enhance GTP[S]-activated phospholipase C (Fig. 7, inset). The activation of phospholipase C by GTP[S] was also inhibited, but was less sensitive to saponin treatment than was the activity stimulated by thrombin or ethanol. At a saponin concentration of 28 μg/ml, the stimulation by ethanol was completely inhibited, but GTP[S] still produced a substantial stimulation of inositol phosphate production. Thus the data provide indirect evidence that ethanol requires an intact receptor-coupled transducing system to stimulate phospholipase C.

Several other approaches were investigated in efforts to dissociate receptor-mediated and GTP[S]-dependent stimulation of phospholipase C. Firstly, pretreatment of intact platelets with chymotrypsin by the method described by McGowan & Detwiler (1986) did not alter subsequent thrombin- or ethanol-induced inositol phosphate formation in permeabilized preparations. Secondly, incubation of saponin-treated platelets with pertussis toxin (20 μg/ml) as described by Brass *et al.* (1986) only partially inhibited thrombin-induced phospholipase C activation and did not alter ethanol-induced stimulation. Thirdly, preincubation of platelets

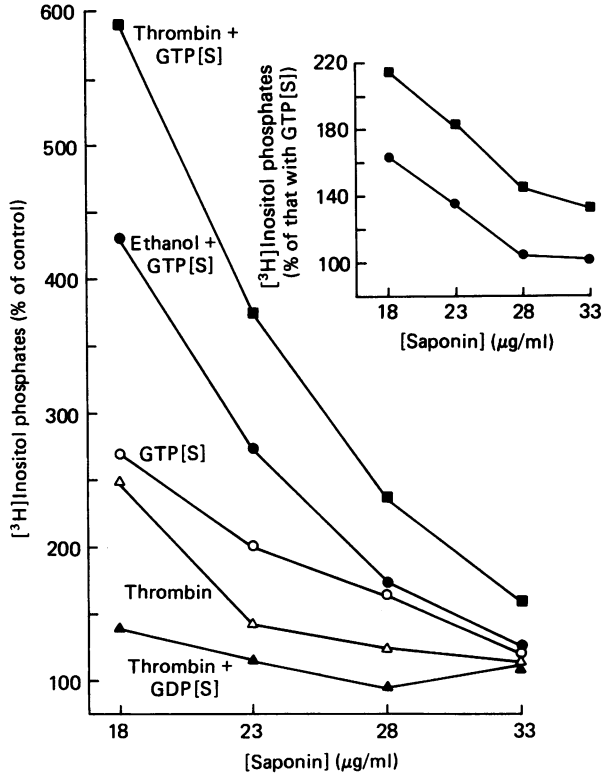

Fig. 7. Inhibition of thrombin- and ethanol-induced phospholipase C activation by saponin

[^{3}H]Inositol-labelled platelets were treated with the indicated concentrations of saponin for 2 min before any additions. Permeabilized platelets were then incubated with thrombin alone (0.1 unit/ml) (△), thrombin plus GDP[S] (0.5 mM) (▲), GTP[S] (50 μM) (○), ethanol (300 mM) plus GTP[S] (●) and thrombin plus GTP[S] (■). GDP[S] was added immediately before addition of saponin. After 15 min, [^{3}H]inositol phosphates were determined as described in the Experimental section. Each point represents the mean of three separate experiments and is expressed as the percentage of control incubations without any additions. Increased concentrations of saponin did not alter inositol phosphate content in control incubations. Inset: inositol phosphate production in response to ethanol plus GTP[S] (●) and thrombin plus GTP[S] (■) is plotted as the percentage of incubations in the presence of GTP[S] alone.

with agents that increased cyclic AMP, i.e. forskolin or prostacyclin, did not inhibit activation of phospholipase C in response to any of the agonists in the permeabilized platelets, even though these agents inhibit the response to ethanol and thrombin in the intact platelet (R. Rubin, unpublished work). Thus many of the factors that affect the response to ethanol in the intact platelets appear not to be operative in the permeabilized system; this supports the interpretation that the effect of ethanol on phospholipase C is exerted directly at the level of the control of phospholipase C in the plasma membrane.

DISCUSSION

The data presented here represent the first direct demonstration of an effect of ethanol on hormone-sensitive phosphoinositide-specific phospholipase C in a broken-cell system. These findings also provide a link with our previous demonstration of the mobilization of intracellular Ca^{2+} and stimulation of polyphosphoinositide turnover by ethanol in intact cells (Hoek *et al.*, 1987; Rubin & Hoek, 1988). The actions of ethanol were similar to those of physiological Ca^{2+}-mobilizing agonists such as thrombin, in that the formation of inositol phosphates and phosphatidic acid required the presence of guanine nucleotides. Ethanol also enhanced the activation of phospholipase C in response to NaF, an agent which is presumed to act directly on the G-protein (Cockcroft, 1987).

The actions of ethanol reported here could represent either a direct effect of ethanol on phospholipase C or an effect on its regulatory proteins. A direct stimulation of phospholipase C by ethanol is unlikely. In the permeabilized platelets, high concentrations of alcohols did not stimulate inositol phosphate release in the absence of guanine nucleotides or NaF. In digitonin-permeabilized or sonicated hepatocytes (Rubin *et al.*, 1987), ethanol (300 mM) was also ineffective in stimulating phospholipase C in response to GTP[S] or 1 mM-$CaCl_2$. It is therefore likely that the action of ethanol is due to a stimulation of phospholipase C via its regulatory components. A precise identification of the locus of action is somewhat limited by the current understanding of the precise structure of these regulatory proteins. Current models of phospholipase C activation by the putative G-proteins are analogous to the control of adenylate cyclase by G_s (Cockcroft, 1987). The binding of Ca^{2+}-mobilizing hormones to receptors would induce the dissociation of G-protein subunits and promote the release of GDP. GTP would then be free to bind to the stimulatory subunits, which could activate phospholipase C concomitantly with the hydrolysis of GTP. A sustained activated state is possible with the non-hydrolysable GTP analogues. Guided by this model, several sites of action for ethanol would be possible. Firstly, ethanol could increase the ability of activated G-protein subunits to stimulate phospholipase C. Secondly, ethanol could promote the dissociation of G-protein subunits. Thirdly, although it is unlikely that ethanol has any specific receptors, ethanol could affect the interaction of receptors with the respective G-proteins. Indeed, in the present study, an effect of ethanol on receptor–G-protein interactions is suggested by the parallel disruption of thrombin- and ethanol-induced activation of phospholipase C by increasing concentrations of saponin (Fig. 7). Our previous studies in permeabilized hepatocytes demonstrated that the disruption of receptor-mediated stimulation of phospholipase C by digitonin also was accompanied by a lack of stimulation by ethanol (Rubin *et al.*, 1987). The mechanism by which these detergents disrupt receptor–phospholipase C coupling is unclear, but it is conceivable that intramembrane protein–protein interactions are sensitive to disruption of the membrane structure. It is also known that the activation of phospholipase C by the activated G-protein is relatively insensitive to cell disruption (Rock & Jackowski, 1987; Baldassare & Fisher, 1986).

Further support for an effect of ethanol on the receptor–G-protein interaction is derived from the finding that ethanol stimulates the maximal activity of phospholipase C, but does not affect the apparent K_d for GTP[S] (Fig. 1). This would suggest that ethanol increases the availability of G-protein subunits for GTP[S]-

mediated stimulation of phospholipase C. Further studies await the isolation and reconstitution of the regulatory proteins involved in phospholipase C activation.

The stimulation of phospholipase C by alcohols is a membrane-interactive phenomenon. In this study, a linear relationship was found between phospholipase C stimulation and the partition coefficients for several short-chain alcohols. Thus the stimulation of phospholipase C appears to correlate directly with the concentration of the respective alcohol within the plasma membrane. The chain length of short-chain alcohols and their relative partitioning into biomembranes also correlate with their ability to disorder lipid bilayer structure ['fluidization'; see Taraschi & Rubin (1985), McCreery & Hunt (1978) and Lyon *et al.* (1981)]. The extent of alcohol partitioning into biomembranes has also been correlated with the stimulation of other membrane-associated processes, such as adenylate cyclase activation in mouse striatal membranes (Luthin & Tabakoff, 1984) and receptor-mediated chloride transport in rat brain synaptoneurosomes (Suzdak *et al.*, 1986). In addition, the general anaesthetic potencies of a wide range of compounds, including alcohols, can be directly related to their lipid solubilities [Lyon *et al.* (1981), and see Miller (1985) for a review]. Thus increased plasma-membrane fluidity may enhance the lateral mobility of G-proteins, thereby increasing the frequency of coupling with either receptors or phospholipase C. However, several recent observations suggest that the effect of ethanol on phospholipase C may not be adequately described by its ability to fluidize membranes. Firstly, hepatocytes obtained from rats that were chronically treated with ethanol do not exhibit a significant decrease in the responsiveness to phospholipase C (J. B. Hoek, unpublished work). Similarly, platelets obtained from the same alcohol-treated rats develop shape change in response to ethanol in a similar manner to control animals (R. Rubin, unpublished work). It is not known, however, whether these platelets are also resistant to the partitioning of ethanol. Secondly, studies on the anaesthetic properties of a wide range of compounds suggest the possibility that these compounds may have a more specific locus of action through their effect on proteins, rather than acting through non-specific membrane-structural effects (Miller, 1985). For example, ethanol could interact with the hydrophobic domains of proteins, an action that would directly influence protein–protein interactions (Franks & Lieb, 1982). Thirdly, as we have indicated previously (Hoek *et al.*, 1987), it is difficult to exclude the possibility that the effects of alcohols could be partly mediated by affecting the dielectric constant of the medium, thereby altering the physical characteristics of the membrane/water interface. Such effects would be qualitatively parallel to their hydrophobicity.

In conclusion, our data provide evidence that the stimulation of hormone-sensitive phospholipase C by ethanol is due to its action at the level of the regulatory components. This property of ethanol may contribute not only to understanding its biological effects on specific cells, but may also be useful as a tool to analyse the role of membrane-structural properties in the control of phospholipase C activity.

We thank Nicholas Kenny for excellent technical assistance and Dr. Andrew P. Thomas for his helpful suggestions. This work was supported by U.S. Public Health Service Grants DK 38461, AA 07186, AA 07211, AA 07215 and AA 07309.

REFERENCES

Authi, K., Evenden, E. & Crawford, N. (1986) Biochem. J. **233**, 709–718
Baldassare, J. J. & Fisher, G. J. (1986) J. Biol. Chem. **261**, 11942–11944
Brass, L. F. & Joseph, S. K. (1985) J. Biol. Chem. **260**, 15172–15179
Brass, L. F., Laposata, M., Banga, H. S. & Rittenhouse, S. E. (1986) J. Biol. Chem. **261**, 16838–16847
Cockcroft, S. (1987) Trends Biochem. Sci. **12**, 75–78
Cooper, R. H., Coll, K. E. & Williamson, J. R. (1985) J. Biol. Chem. **260**, 3281–3288
Franks, N. P. & Lieb, W. R. (1982) Nature (London) **300**, 487–493
Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
Hoek, J. B., Thomas, A. P., Rubin, R. & Rubin, E. (1987) J. Biol. Chem. **262**, 682–691
Hoek, J. B., Rubin, R. & Thomas, A. P. (1988) Biochem. J. **251**, 865–871
Jolles, J., Wirtz, K. W. A., Schotman, P. & Gispen, W. H. (1979) FEBS Lett. **105**, 110–114
Lapetina, E. G. (1986) Biochim. Biophys. Acta. **884**, 219–224
Lapetina, E. G., Silio, J. & Ruggiero, M. (1985) J. Biol. Chem. **260**, 7078–7083
Luthin, G. R. & Tabakoff, B. (1984) J. Pharmacol. Exp. Ther. **228**, 579–587
Lynch, C. J., Charest, R., Bocckino, S. B., Exton, J. H. & Blackmore, P. F. (1985) J. Biol. Chem. **260**, 2844–2851
Lyon, R. C., McComb, J. A., Schreurs, J. & Goldstein, D. B. (1981) J. Pharmacol. Exp. Ther. **218**, 669–675
McCreery, M. J. & Hunt, W. A. (1978) Neuropharmacology **17**, 451–461
McGowan, E. B. & Detwiler, T. C. (1986) J. Biol. Chem. **261**, 739–746
Miller, K. W. (1985) Int. Rev. Neurobiol. **27**, 1–60
Rock, C. O. & Jackowski, S. (1987) J. Biol. Chem. **262**, 5492–5498
Rubin, R. & Hoek, J. B. (1988) Biochem. Pharmacol., in the press
Rubin, R., Thomas, A. P. & Hoek, J. B. (1987) Arch. Biochem. Biophys. **256**, 29–38
Rubin, R., Ponnappa, B. C., Thomas, A. P. & Hoek, J. B. (1988) Arch. Biochem. Biophys. **260**, 480–492
Siess, W. & Lapetina, E. G. (1986) FEBS Lett. **207**, 53–57
Suzdak, P. D., Schwartz, R. D., Skolnick, P. & Paul, S. M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4071–4075
Taraschi, T. F. & Rubin, E. (1985) Lab. Invest. **52**, 120–131
Thomas, A. P., Alexander, J. & Williamson, J. R. (1984) J. Biol. Chem. **258**, 5716–5725
Tsien, R. Y. (1981) Nature (London) **290**, 527–528
Zucker, M. B. & Nachmias, V. T. (1985) Arteriosclerosis **5**, 2–18

Received 2 February 1988/29 March 1988; accepted 5 April 1988