Ethanol-induced phospholipase C activation is inhibited by phorbol esters in isolated hepatocytes

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Ethanol causes a transient activation of the phosphoinositide-specific phospholipase C in intact hepatocytes and mimics the action of receptor-mediated agonists [Hoek, Thomas, Rubin & Rubin (1987) *J. Biol. Chem.* **262**, 682–691]. Preincubation of the hepatocytes with phorbol esters which activate protein kinase C prevented this effect of ethanol: phorbol ester treatment inhibited the ethanol-induced phosphorylase activation, the increase in intracellular free Ca^{2+} concentrations measured in quin 2-loaded hepatocytes, and the changes in concentrations of inositol phosphates, phosphoinositides and phosphatidic acid. Several lines of evidence indicate that these effects were mediated by protein kinase C. Phorbol esters acted in a concentration range where they activate protein kinase C; phorbol esters that do not activate protein kinase C were not effective in inhibiting the effects of ethanol. The permeant diacylglycerol oleoyl-acetyl-glycerol also inhibited the effects of ethanol, but other diacylglycerols were not effective in the intact cells. The inhibition of ethanol-induced Ca^{2+} mobilization by phorbol esters was prevented by preincubating the cells with the protein kinase C inhibitors 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) and sphingosine. H7 also enhanced the Ca^{2+} mobilization induced by ethanol in cells that were not pretreated with phorbol esters, indicating that the transient nature of the ethanol-induced Ca^{2+} mobilization may be due to an activation of protein kinase C caused by the accumulation of diacylglycerol. These data support a model whereby ethanol activates the phosphoinositide-specific phospholipase C, possibly by affecting receptor–G-protein–phospholipase C interactions in the membrane.

INTRODUCTION

In a series of recent studies in hepatocytes [1–4], we reported that ethanol addition caused a transient activation of the hormone-sensitive phosphoinositide-specific phospholipase C, with the consequent formation of inositol phosphates and phosphatidic acid, the mobilization of intracellular Ca^{2+} and the activation of Ca^{2+} -dependent enzymes, specifically phosphorylase *a*. The concentration range in which ethanol was active and the fact that other hydrophobic solvents could exhibit similar responses indicated that this effect was not due to the specific interaction of ethanol with a particular receptor, but was a consequence of the interaction of these agents with the plasma membrane. Pretreatment of liver cells with phorbol esters such as TPA prevents phospholipase C activation and Ca^{2+} mobilization induced by α_1 -adrenergic agonists [5–7] and by glucagon [8,9]. Phorbol ester treatment of liver cells also inhibits the effect of low concentrations of other hormones that stimulate phospholipase C, e.g. vasopressin or angiotensin II, but the responses to saturating concentrations of the latter agonists are unaffected [5–7]. Some of these effects could be explained by an activation of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase [10], leading to a decrease in the steady-state concentration of $\text{Ins}(1,4,5)\text{P}_3$. However, studies on various intact cells [5,6] and membrane preparations [11,12] demonstrate that the activation of phospholipase C itself is affected by phorbol ester treatment, indicating interference at an early step in the signal-transduction

sequence. There is evidence from different cell types that either the receptors or the G-protein(s) that control agonist-induced phospholipase C activation can be targets of phorbol ester treatment [5,6,11,12].

In the present study, we report that phorbol esters which activate protein kinase C also inhibit the action of ethanol on the phosphoinositide-specific phospholipase C. Inhibitors of protein kinase C prevent the action of the phorbol esters. Furthermore, these inhibitors also enhance the Ca^{2+} mobilization in response to ethanol in cells that are not pretreated with phorbol esters, providing evidence in support of a feedback regulation of phospholipase C activation by endogenously generated diacylglycerol. A preliminary account of some of these findings has appeared in abstract form [13].

METHODS AND MATERIALS

Preparations and incubation conditions

Isolated hepatocytes were prepared by collagenase perfusion from fed male Sprague–Dawley rats exactly as described in previous publications [1,3]. Incubation conditions for assaying phosphorylase activity, for labelling the cells with *myo*-[^3H]inositol or [^{32}P]P_i and measuring phosphoinositides and inositol phosphates, for loading with quin 2 and measuring changes in cellular free Ca^{2+} concentrations have all been described previously [1].

Abbreviations used: $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; TPA, 12-*O*-tetradecanoyl-4 β -phorbol 13-acetate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; $\text{PtdIns}4\text{P}$, phosphatidylinositol 4-phosphate; G-protein, guanine nucleotide regulatory protein; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; DMSO, dimethyl sulfoxide.

Fig. 1. Effect of TPA on phosphorylase activation by ethanol and A23187

Isolated hepatocytes were preincubated for 25 min before the addition of TPA (100 nM from a stock solution in DMSO). Control cells received an equivalent amount of DMSO. Then 5 min later, ethanol (100 mM) or A23187 (20 μ M from a stock solution in DMSO) was added, and samples were taken at the time points indicated for the determination of phosphorylase activity. Results are representative of three similar experiments.

Assays

Phosphorylase activity was measured by a modification of the method of Gilboe *et al.* [14], after extraction of the cells with an ice-cold buffered EDTA/NaF medium containing 0.2 mM-digitonin (see [1]). [3 H]Inositol phosphates were measured in neutralized HClO₄ extracts after separation by h.p.l.c. by a modification of the method of Irvine *et al.* [15] as previously described [1]. 32 P-labelled phosphoinositides and phosphatidic acid were determined after extracting the cells with chloroform/methanol/HCl and separation of the lipids obtained from the chloroform phase by t.l.c. on oxalate-treated silica gel 60 (see [1] for details). Protein was determined by the biuret method.

Presentation of data

Experiments are presented either as the results obtained from a single experiment, representative of at least three similar ones, or as the mean $\pm$ S.E.M. for three or more experiments. Statistical significance was calculated by Student's *t* test.

Materials

myo-[2- 3 H]Inositol (15 Ci/mmol in 95% ethanol) was purchased from American Radiolabelled Chemicals. The ethanol was removed by evaporation and the labelled compound was treated before use with a small amount of Dowex 1-X8 anion-exchange resin (formate form; 200–400 mesh) (Bio-Rad) to remove impurities [1]. GTP[S] was obtained from Boehringer-Mannheim. H7 was obtained from Seikagaku America (St. Petersburg, FL, U.S.A.) and used as a solution in DMSO. Other chemicals and biochemicals were obtained from Sigma Chemical Co. or Fisher Scientific Co.

RESULTS

In isolated hepatocytes, a rapid increase in cytosolic free Ca²⁺ is induced by the addition of ethanol in the

concentration range 25–500 mM which can be detected by activation of phosphorylase or, in quin 2-loaded cells, by the increase in the Ca²⁺-specific fluorescence response. In Fig. 1, the time course of Ca²⁺ mobilization, measured by phosphorylase activation, was followed after the addition of 100 mM-ethanol or 20 μ M ionophore A23187. Ca²⁺ mobilization by ethanol was almost completely inhibited by pretreating the cells for 5 min with 100 nM-TPA, whereas TPA had no effect on the Ca²⁺ mobilization by the ionophore. This latter finding is in agreement with the data of Lynch *et al.* [5], but contrasts with a report by Van de Werve *et al.* ([16]; see also [17]).

In Fig. 2, an experiment is shown with quin 2-loaded cells. Again, the Ca²⁺ mobilization induced by ethanol (300 mM) was completely inhibited by pretreating the cells for 2 min with 40 nM-TPA (Fig. 2d), whereas the ionomycin-induced Ca²⁺ increase was unaffected. The effect of TPA was not immediate; after 30 s preincubation the Ca²⁺ transient was inhibited by approx. 50% (Fig. 2c). When TPA was added together with, or after, the ethanol, the peak Ca²⁺ concentration was not significantly affected (Fig. 2b), and TPA added after the Ca²⁺ response had reached its peak had little or no further effect (results not shown). This behaviour is different from the phospholipase C activation by other agonists, which could be reversed even when TPA was added after the Ca²⁺ mobilization had reached its peak [5–9]. It should be pointed out that the Ca²⁺ response to ethanol is much more transient than that induced by other agonists, as we observed previously [1,2,4].

In quin 2-loaded cells, 40 nM-TPA completely inhibited ethanol-induced Ca²⁺ mobilization, independent of the ethanol concentration. A half-maximal inhibition of the rate of Ca²⁺ mobilization was found at approx. 5–10 nM-TPA, and the effect of suboptimal concentrations of TPA could not be overcome by increasing the concentration of ethanol. This is shown in Fig. 3, where the effect of TPA was found to be similar at ethanol concentrations of 150 mM or 300 mM. By contrast, the

Fig. 2. Effect of TPA on ethanol-induced Ca^{2+} mobilization in quin 2-loaded hepatocytes

Cells were loaded with quin 2 as described [1] and the fluorescence signal was measured at a wavelength pair of 339 nm and 495 nm. After a 10 min preincubation in the presence of 4-methylpyrazole (15 mM) (which is added to prevent interference by ethanol-induced NAD(P) redox changes; see [1]), 300 mM-ethanol was added to all incubations. TPA (40 nM) was added where indicated, 2 s (b), 30 s (c) or 2 min (d) before ethanol. In the control (a), an equivalent volume (4 mM) of DMSO was added 2 min before ethanol. Ionomycin (4 μM) was added to determine the fluorescence of saturating cytosolic Ca^{2+} concentrations.

effect of TPA pretreatment on phosphorylase activation was markedly dependent on the concentration of ethanol used. As shown in Fig. 4, the effect of TPA was to cause a shift in the dose-response curve for ethanol; the ethanol concentration to give half-maximal stimulation was increased from about 70 mM in the absence of TPA to 140 mM-ethanol at 10 nM-TPA and > 200 mM-ethanol at 100 nM-TPA. Higher concentrations of TPA had no

further inhibitory effect, indicating that these effects are not due to a simple competitive relationship between TPA and ethanol. Possible reasons for these different sensitivities to TPA treatment between Ca^{2+} mobilization and phosphorylase activation are considered in the Discussion section.

The ethanol-induced Ca^{2+} mobilization is secondary to the activation of phospholipase C and the formation of $\text{Ins}(1,4,5)\text{P}_3$ [1]. We therefore investigated whether TPA also exerts its effects on the breakdown of polyphosphoinositides and the formation of inositol phosphates in response to ethanol and phenylephrine. In the experiment of Table 1, isolated hepatocytes were pre-labelled with [^3H]inositol for 90 min, and inositol phosphates were separated from the cell extracts by h.p.l.c. as described previously [1]. A 30 s exposure to ethanol (300 mM) or phenylephrine (10 μM) increased $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4)\text{P}_3$ and inositol biphosphate, in agreement with previous data from this and other laboratories [1,5,6]. Treatment of the cells with 100 nM-TPA for 2 min completely inhibited the formation of all inositol phosphates, indicating an inhibition of phospholipase C activation induced by either ethanol or the α_1 -agonist. TPA also partially suppressed the amounts of inositol phosphates in the absence of the agonists. The mechanism of this effect is not known; it may reflect an activation of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase by TPA [10]. The effects of ethanol on the amounts of polyphosphoinositides and phosphatidic acid measured in ^{32}P -labelled cells are also antagonized by TPA pretreatment (Table 2). This is not readily detectable in $\text{PtdIns}(4,5)\text{P}_2$, since changes in this phospholipid are variable and difficult to detect, as reported previously [1,4]; however, the ethanol-induced changes in the amounts of $\text{PtdIns}4\text{P}$ and phosphatidic acid were consistently inhibited by phorbol ester pretreatment. Taken together, the data of Tables 1 and 2 provide evidence that the phorbol ester pretreatment inhibits the ethanol-induced activation of phospholipase C. Moreover, the activation of phosphatidylinositol kinase by ethanol, which is reflected in the increase in $\text{PtdIns}4\text{P}$ [4], is also prevented by phorbol ester treatment.

We have tried to find direct evidence for an effect of TPA treatment on phospholipase C activation in studies with hepatocytes permeabilized with a minimal concentration of digitonin (see [3]). In this system, phospholipase C is no longer responsive to receptor-mediated agonists, but can be activated 2–3-fold by GTP[S] (in the presence of 200 nM free Ca^{2+}); half-maximal activation occurs at 0.6 μM -GTP[S] [3]. A 5 min pretreatment of the cells with 100 nM-TPA before permeabilization had only a marginal effect on the capacity of GTP[S] to stimulate the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$: in the untreated permeabilized cells, 1 μM -GTP[S] stimulated inositol phosphate production by $73 \pm 8\%$, whereas in the TPA-pretreated cells a stimulation of $61 \pm 4\%$ was found (mean $\pm$ S.E.M. for three incubations; difference not significant at $P < 0.05$). Thus the G-protein-mediated activation of phospholipase C in these permeabilized hepatocytes appears to be relatively unaffected by TPA treatment.

The TPA concentration required for half-maximal inhibition of the ethanol-induced Ca^{2+} mobilization in intact hepatocytes was 5–10 nM, as measured both by phosphorylase activation and by Ca^{2+} mobilization in quin 2-loaded cells (results not shown). This concen-

Fig. 3. Inhibition by TPA of Ca^{2+} mobilization induced by 150 mM- and 300 mM-ethanol in quin 2-loaded hepatocytes

Incubation conditions were as described for Fig. 2. TPA was added in the concentrations indicated 2 min before the addition of 150 mM- (a-c) or 300 mM- (d-f) ethanol.

Table 1. Antagonism by phorbol ester of ethanol- and phenylephrine-induced inositol phosphate formation

Cells were labelled with [^3H]inositol (5 $\mu\text{Ci}/\text{mg}$) for 90 min before incubation, as described in the Methods and materials section and in [1]. TPA was added where indicated, 2 min before ethanol or phenylephrine, and samples for analysis of inositol polyphosphates by h.p.l.c. were taken 30 s after these agents were added. Results are means $\pm$ S.E.M. for four separate experiments each done in triplicate. * $P < 0.05$; ** $P < 0.01$.

Additions	^3H in inositol phosphates (% of control)		
	Ins(1,4,5) P_3	Ins(1,3,4) P_3	Inositol bispophosphate
300 mM-Ethanol	157.1 $\pm$ 17.2*	197.1 $\pm$ 19.4**	139.3 $\pm$ 11.9*
10 μM -Phenylephrine	159.1 $\pm$ 16.6*	193.5 $\pm$ 22.7**	131.0 $\pm$ 14.1
100 nM-TPA	77.9 $\pm$ 5.9*	82.9 $\pm$ 13.4	72.7 $\pm$ 8.2*
100 nM-TPA + 300 mM-ethanol	93.8 $\pm$ 8.4	101.2 $\pm$ 7.5	101.1 $\pm$ 22.0
100 nM-TPA + 10 μM -phenylephrine	90.9 $\pm$ 7.0	101.5 $\pm$ 10.1	109.1 $\pm$ 15.4

tration is similar to that required for half-maximal inhibition of the Ca^{2+} mobilization by phenylephrine; glucagon or low concentrations of vasopressin ([5-9]; J. B. Hoek, unpublished work). TPA activates protein kinase C over the same concentration range [5], suggesting that its effects are mediated through that enzyme. In agreement with this interpretation, other phorbol esters that activate protein kinase C, e.g. phorbol dibutyrate, had similar effects, but phorbol derivatives that do not activate protein kinase C (4 β -phorbol, phorbol 13-acetate) did not inhibit the mobilization of Ca^{2+} by ethanol or hormones (results not shown). We also tested the effects of diacylglycerols that could penetrate through

the cell membrane and activate protein kinase C directly. As shown in Fig. 5, ethanol-induced phosphorylase activation was also inhibited by oleoylacetyl-glycerol, although relatively high concentrations were required. In previous studies, Cooper *et al.* [6] reported that inhibition of phenylephrine-induced Ca^{2+} mobilization by this compound also required concentrations up to 200 μM . No significant effects were observed with diacylglycerols with medium acyl-chain lengths, e.g. dioctanoyl- and didecanoyl-glycerol, which have been reported to activate protein kinase C in platelets [18]. It is possible that the diacylglycerols do not accumulate to a sufficiently high concentration in the cell to be effective in activating

Fig. 4. Effect of TPA on ethanol dose-response curve for phosphorylase activation

Incubations were carried out as described for Fig. 1. Incubation time after ethanol addition was 30 s.

protein kinase C, e.g., owing to a lower rate of penetration across the plasma membrane or a higher rate of metabolism of the diacylglycerols in the hepatocytes as compared with the platelets.

Different inhibitors of protein kinase C were tested for their ability to prevent the effect of TPA on ethanol-induced Ca^{2+} mobilization. Chlorpromazine (50–500 μM) and dibucaine (500 μM) which inhibit isolated rat brain protein kinase C [19] were unable to prevent the actions of TPA; instead, high concentrations of these agents induced a significant mobilization of Ca^{2+} and partly suppressed the effect of ethanol in quin 2-loaded hepatocytes (results not shown). Polymyxin B sulphate, a protein kinase C inhibitor [20] which has been reported to inhibit protein kinase C-mediated metabolic effects in liver cells [21], did not influence the ethanol-induced Ca^{2+} mobilization or the antagonism thereof by TPA. Some other inhibitors, however, were effective in this respect. As shown in Fig. 6, the inhibition of ethanol-induced Ca^{2+} mobilization by TPA was completely prevented by preincubating the hepatocytes for 10 min with 400 μM -H7, a protein kinase inhibitor with some specificity for protein kinase C [22]. H7 in this concentration range caused a fluorescence-quenching artefact and a slow upward drift in the background fluorescence response in a hepatocyte suspension; however, at the wavelength pair used in these studies (339 nm and 495 nm), the Ca^{2+} -dependent fluorescence response, calibrated by the addition of excess EGTA after permeabilization of the cells with digitonin in a Ca^{2+} -containing medium, was not significantly decreased by the presence of H7. Sphingosine (200 μM) could similarly prevent the effect of TPA on ethanol-induced Ca^{2+} mobilization and did not exhibit the fluorescence-

Table 2. Effect of TPA pretreatment on ethanol-induced changes in phosphatidic acid and phosphoinositide contents

Cells were preincubated for 90 min with [^{32}P]P_i before incubation with or without 100 nM-TPA. Ethanol (300 mM) was added 5 min afterwards, and incubations were continued for 30 s before extraction with chloroform/methanol/HCl for the determination of radioactivity incorporated into phospholipids. Results are means $\pm$ S.E.M. for five individual experiments, done in triplicate. * $P < 0.02$ compared with control; † $P < 0.01$ compared with condition without TPA; ‡ $P < 0.05$ compared with condition without TPA.

Additions	^{32}P radioactivity in lipid (% of control)		
	PtIns4P	PtdIns(4,5)P ₂	Phosphatidic acid
Ethanol	110 $\pm$ 1.2*	103 $\pm$ 1.7	111 $\pm$ 1.0*
Ethanol + TPA	99 $\pm$ 0.9†	102 $\pm$ 0.8	105 $\pm$ 2.1‡

Fig. 5. Effect of oleoylacylglycerol on ethanol-induced phosphorylase activation

Incubation conditions were as in the experiment of Fig. 1. Cells were preincubated for 2 min with the indicated concentrations of OAG before addition of ethanol (100 mM).

quenching artefact (results not shown); however, the addition of sphingosine at this concentration caused a transient increase in the Ca^{2+} concentration in the quin 2-loaded hepatocytes, which interfered with the subsequent response to ethanol.

Interestingly, pretreating the cells with H7 also had an effect on the ethanol-induced Ca^{2+} transient in the absence of TPA. As shown in Fig. 6(c), the decay of the ethanol-induced Ca^{2+} mobilization was significantly slower after pretreating the cells with H7. At lower concentrations of ethanol, H7 pretreatment also enhanced the Ca^{2+} mobilization and increased the peak

Fig. 6. Effect of H7 pretreatment on ethanol-induced Ca^{2+} mobilization in quin 2-loaded cells in the presence and the absence of TPA

Experimental conditions were as in Fig. 2, except that cells were preincubated with $400\ \mu\text{M}$ -H7 for 10 min in (c) and (d). TPA ($100\ \text{nM}$), ethanol ($300\ \text{mM}$) and ionomycin ($15\ \mu\text{M}$) were added as indicated.

free Ca^{2+} concentration induced by ethanol. Similar results were obtained with sphingosine (results not shown). These observations indicate that protein kinase C also plays a role in regulating the response of the cell to ethanol in the absence of TPA.

DISCUSSION

Previous studies in hepatocytes and other cell types have demonstrated that phorbol esters inhibit several receptor-mediated processes which depend on the activation of phospholipase C, including polyphosphoinositide breakdown, inositol phosphate formation, intracellular Ca^{2+} mobilization, and activation of Ca^{2+} -dependent enzymes [5–9,11,12]. As shown in the present paper, the activation of phospholipase C by ethanol is similarly affected by phorbol ester pretreatment of

hepatocytes, even though ethanol presumably does not act by binding to a specific receptor in the membrane.

In liver cells, the sensitivity to phorbol ester treatment differs for α_1 -adrenergic agonists and for vasopressin or angiotensin II; the response to the α_1 -agonists can be completely inhibited irrespective of the agonist concentration, whereas higher hormone concentrations can overcome the effect of phorbol ester treatment on responses to vasopressin and angiotensin II [5–7]. The effect of ethanol concentration on the inhibition of Ca^{2+} mobilization by TPA (Figs. 3 and 4) appears to be less clearly defined. Whereas the inhibition of phosphorylase activation by TPA could be overcome by high concentrations of ethanol, Ca^{2+} mobilization in quin 2-loaded cells was inhibited at all concentrations of ethanol examined. This difference is not due to the Ca^{2+} -buffering action of quin 2 in the cells, since similar observations were made in fura 2-loaded cells, which contained a much lower concentration of indicator (results not shown). Moreover, the formation of $\text{Ins}(1,4,5)\text{P}_3$ was inhibited by TPA pretreatment, even at $300\ \text{mM}$ -ethanol (Table 1). A possible explanation of this apparent discrepancy is that phosphorylase kinase was activated directly by high concentrations of ethanol, similar to the activation of muscle phosphorylase kinase by ethanol reported by Singh & Wang [23]. In a previous report [1], we observed a significant stimulation of phosphorylase kinase in broken liver cells by ethanol concentrations in excess of $200\ \text{mM}$. Alternatively, high concentrations of ethanol could induce some formation of cyclic AMP. In several cell types, ethanol has been shown to activate adenylate cyclase [24–26]. Cyclic AMP-induced activation of phosphorylase kinase is not affected by TPA pretreatment ([5,7,16]; J. B. Hoek, unpublished work). Thus the data of Figs. 3 and 4 indicate that the activation of phosphorylase kinase in TPA-treated cells by high concentrations of ethanol is independent of Ca^{2+} ; the inhibition by TPA of ethanol-induced phospholipase C activation and the consequent mobilization of Ca^{2+} cannot be overcome by increasing the ethanol concentration over the range studied here.

The actions of phorbol esters on phospholipase C activation appear to be mediated by protein kinase C-dependent phosphorylation: the dose-response curve for TPA and the structure-activity relationship of different phorbol esters agree with their ability to activate protein kinase C, and the effect of TPA can be partly mimicked by high concentrations of oleoylacylglycerol. Moreover, H7 and sphingosine, inhibitors of protein kinase C, prevent the action of TPA. The target of protein kinase C activity is not clear. The differential sensitivity of agonists to phorbol ester treatment suggests a response at the receptor level. In other systems [27,28], there is, in fact, direct evidence demonstrating the phosphorylation of receptors induced by protein kinase C or phorbol ester treatment. Recently, however, the G-proteins that control phospholipase C activation have been suggested as potential targets of protein kinase C activity. In several systems, GTP[S]-induced phospholipase C activity was found to be inhibited by phorbol ester treatment or protein kinase C [11,12]. In intact hepatocytes, the phospholipase C activation by AlF_4^- was shown to be inhibited by phorbol ester pretreatment [8]; this agent is presumed to act directly on the G-protein [29]. Our finding that ethanol-induced activation of phospholipase C is also prevented by TPA treatment can be interpreted

as further support for a site of action of protein kinase C beyond the receptor level. Since a variety of different agonists exhibit this sensitivity to TPA, the target of protein kinase C action may be shared by all these agents. However, in hepatocytes permeabilized by digitonin we were unable to detect a significant inhibition of GTP[S]-induced phospholipase C activity. In this permeabilized cell preparation, we reported previously [3] that ethanol did not stimulate phospholipase C. We have interpreted these data to indicate that the action of ethanol is dependent on the integrity of the receptor-G-protein-phospholipase C complex in the membrane. This interpretation is supported by our observation that in saponin-permeabilized platelets, which maintain receptor-mediated phospholipase C activation, ethanol could stimulate $\text{Ins}(1,4,5)\text{P}_3$ formation in a GTP[S]-dependent manner (R. Rubin & J. B. Hoek, unpublished work). In accordance with this model, TPA treatment may affect the interaction between G-proteins and receptors in such a way as to lock the G protein in an inactive conformation. In principle, this could be due to a phosphorylation of either the receptor or the G-protein. It would result in a lack of stimulation of phospholipase C, even when GTP analogues or fluoride are used as activators, unless the receptor-G-protein interaction is disrupted by other factors, e.g. as a consequence of the permeabilization procedure.

The physiological significance of the inhibition by phorbol esters of phospholipase C is not well understood, but is usually interpreted in terms of feedback regulation of the receptor-mediated signal-transduction pathways [5,6]. So far, there has been little direct evidence that this feedback inhibition operates in the intact cell with protein kinase C activated by diacylglycerol formed from phospholipase C. Our data give an indication in support of such a model. We have found previously [1] that the ethanol-induced activation of phospholipase C and the consequent Ca^{2+} mobilization is transient, with $\text{Ins}(1,4,5)\text{P}_3$ formation reaching a maximum about 20 s after ethanol addition and declining to baseline values within about 2 min. The experiment shown in Fig. 6 demonstrates that pretreatment of the cells with the protein kinase C inhibitor H7 not only prevents the inhibitory action of TPA, but also enhances Ca^{2+} mobilization in response to ethanol in the control cells. A possible interpretation of these data is that the accumulation of diacylglycerol in response to phospholipase C activation stimulates protein kinase C, which then feeds back to give a partial inhibition of the activated complex. Thus protein kinase C may limit the extent of Ca^{2+} mobilization by ethanol even in the absence of phorbol esters, and activation of protein kinase C by TPA after ethanol has acted would not cause much further inhibition (cf. Fig. 2). If this interpretation is correct, it suggests a feedback control function mediated by protein kinase C which could have important longer-term consequences for the function of the cell after one or more exposures to ethanol.

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