The effect of ethanol on receptor-mediated phospholipase C-linked signal transduction processes was investigated in isolated rat hepatocytes. Pretreatment of the cells with ethanol (6-300 mM) markedly inhibited a subsequent stimulation of phospholipase C by vasopressin, angiotensin II, or epidermal growth factor. By contrast, the effects of the α-receptor agonist phenylephrine and of glucagon were not affected by ethanol pretreatment. Ethanol inhibited the agonist-induced decrease in polyphosphoinositides, the formation of inositol phosphates, and the increase in cytosolic free Ca²⁺ levels, as detected with the intracellular Ca²⁺ indicator indo-1. The effects of ethanol were concentration dependent and were pronounced at low concentrations of agonists but were not significant at saturating levels. Pretreatment of the cells with the protein kinase C (PKC) inhibitor H₂O₂ partly prevented the effect of ethanol on vasopressin-induced phospholipase C activation. By contrast, pretreatment of the cells with (R)⁻-adenosine cyclic 3'5'-phosphorothioate ([(R)⁻]-cAMP-S), a competitive inhibitor of protein kinase A, potentiated the inhibitory effect of ethanol on the Ca²⁺ mobilization by vasopressin. (R)⁻-cAMP-S similarly potentiated the inhibition of phospholipase C by the protein kinase C-activating phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA). The kinase A inhibitor also made the Ca²⁺ mobilization by phenylephrine sensitive to ethanol, indicating that the formation of cAMP in the cells played a role in suppressing the sensitivity to ethanol. Pretreatment of the cells with ethanol enhanced the inhibitory effects of TPA on the vasopressin-induced phospholipase C activation at all concentrations of the hormone; however, these synergistic effects were prevented when TPA was added prior to ethanol, a condition that prevents the activation of phospholipase C by ethanol. The data indicate that ethanol causes desensitization of the receptor-mediated phospholipase C secondary to the ethanol-induced activation of phospholipase C and activation of protein kinase C. Ethanol treatment also affects the sensitivity of the phospholipase C system to control by protein kinases A and C. The data indicate that ethanol can affect the control of intracellular signal transduction processes in liver cells under physiologically relevant conditions.

In a recent series of studies in isolated hepatocytes and other cells (1-6), we have demonstrated that ethanol, in concentrations of 25-500 mM, can cause a rapid but transient activation of the hormone-sensitive, phosphoinositide-specific phospholipase C. The consequent rise in inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), which is also transient, causes a mobilization of Ca²⁺ from intracellular stores which can be detected with intracellular fluorescent Ca²⁺ indicators such as quin-2, fura-2, or indo-1 (1). The rise in cytosolic free Ca²⁺ levels activates Ca²⁺-dependent enzymes, e.g. phosphorylase kinase in liver cells (1, 3) and myosin light chain kinase in human platelets (2). The second product of polyphosphoinositide breakdown is diacylglycerol, which can activate protein kinase C. A protein kinase C-mediated phosphorylation of intracellular proteins by ethanol was demonstrated directly in human platelets (2). We have shown recently (3) that the protein kinase inhibitor H₂O₂, which acts as a preferential kinase C inhibitor in many cells (7), enhanced the phospholipase C activation by ethanol in hepatocytes. These findings indicate that the protein kinase C activation by ethanol may be responsible for the transient nature of the ethanol-induced Ca²⁺ mobilization in hepatocytes by feedback inhibition of the hormone-sensitive phospholipase C.

The effects of several agonists that activate phospholipase C by a receptor-mediated mechanism (vasopressin, angiotensin II, EGF, phenylephrine, glucagon) are also inhibited by protein kinase C (8-12). This raises the question of whether ethanol pretreatment of hepatocytes can also inhibit the response to these agonists.

In the present study, we demonstrate that ethanol pretreatment does inhibit the Ca²⁺ mobilization induced by several hormones that activate phospholipase C in isolated hepatocytes. This inhibition has characteristics of a heterologous desensitization of these receptor-mediated processes by ethanol. We provide evidence that the effect of ethanol is due, at least in part, to an activation of protein kinase C. However, ethanol also affected receptor-mediated responses by a mechanism distinct from protein kinase C since it acted synergistically with phorbol esters that activate protein kinase C. At physiological hormone levels, a marked effect of ethanol could be demonstrated at concentrations that were well within the

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The abbreviations used are: Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; Ins(1,3,4)P₃, inositol 1,3,4-trisphosphate; InsP₄, myo-inositol bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(5)P, phosphatidylinositol 5-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; HPLC, high performance liquid chromatography; iso-1 AM, indol-1 pentaacetoxymethylster; TPA, 12-0-tetradecanoylphorbol-13-acetate; H₂O₂, 1-(5-isoquinolinylsulfonfyl)-2-methylpiperazaine; G-protein, guanine nucleotide regulatory protein; TPEN, tetrakis-N,N',N''-(2-pyridylmethyl)-ethylendiamine; EGF, epidermal growth factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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A. 0.1 nM

B. 0.4 nM

C. 40 nM

FIG. 1. Effect of TPA and ethanol on the vasopressin-induced increase in cytosolic Ca\(^{2+}\). Isolated hepatocytes were loaded with indo-1 and incubated at 37 °C in Krebs-Ringer medium containing 2 mM CaCl\(_2\), 10 mM Hepes, 0.2% bovine serum albumin. The fluorescence signal was measured at a wavelength pair of 340 nm/395 nm. After a 3–5-min preincubation in the presence of 10 μM TPEN and 10 mM 4-methylpyrazole, cells were stimulated with 0.1 nM (A), 0.4 nM (B), or 40 nM (C) vasopressin. Ethanol (middle traces) and TPA (bottom traces) were added as indicated, 2–3 min prior to vasopressin. CaCl\(_2\) (2 mM) and ionomycin (20 μM) were added at the end of each incubation to determine the fluorescence of saturating cytosolic Ca\(^{2+}\) concentrations. Note the increase in chart speed in Fig. 1C just prior to vasopressin addition.

physiologically relevant range. By contrast, pretreatment with ethanol did not affect the Ca\(^{2+}\) mobilization induced by phenylephrine or glucagon; instead, these agonists appeared to inhibit the actions of ethanol on phosphoinositide turnover. Evidence is provided that the production of cAMP and the consequent activation of protein kinase A are involved in preventing these effects of ethanol.

The interactions of ethanol with the phosphoinositide-linked signaling processes demonstrate the existence of a hitherto unsuspected susceptibility of cellular control processes to ethanol which may have significant physiological implications. A preliminary report of some of these findings has appeared (13).

EXPERIMENTAL PROCEDURES

Preparations and Incubation Conditions—Isolated hepatocytes were prepared by collagenase perfusion from fed male Sprague-Dawley rats and incubated in a standard Krebs-Ringer medium containing 2 mM CaCl\(_2\), 10 mM Hepes, and 2% bovine serum albumin, as described in previous publications (1, 3). Where indicated, cells were preincubated for 90 min for labeling with myo-[\(^{3}H\)]inositol or [\(^{32}P\)]Pi, for the determination of inositol phosphate and polyphosphoinositide levels, respectively (1). Changes in intracellular Ca\(^{2+}\) levels were studied in hepatocytes loaded with the intracellular Ca\(^{2+}\) indicator indo-1. Cells were loaded with the indicator by a 40-min incubation with indo-1 AM (5 μM) in the presence of 0.0012% Pluronic F-127 followed by a 20-min incubation in the standard incubation buffer containing 0.2% bovine serum albumin. Indo-1-loaded cells were kept on ice and washed just prior to use to remove any dye that had leaked from cells. Incubations were carried out in the same medium in a cuvette of a Perkin Elmer MPF-44B spectrofluorometer at a temperature of 37 °C. Indo-1 fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 395 nm. Emission ratio measurements cannot be carried out with this system, but indo-1 has the advantage, at this wavelength pair, that interfer-
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RESULTS

Effects of Ethanol on Agonist-induced Ca\(^{2+}\) Mobilization—
The vasopressin-induced activation of polyphosphoinositide-specific phospholipase C in intact hepatocytes causes an increase in cytosolic free Ca\(^{2+}\) levels by releasing Ca\(^{2+}\) from intracellular stores and activating Ca\(^{2+}\) influx from the extracellular medium. In agreement with earlier studies by Lynch et al. (8) and Cooper et al. (9), we found that pretreatment of the cells with the phorbol ester TPA inhibited the Ca\(^{2+}\) mobilization induced by submaximal levels of vasopressin (0.1 or 0.4 nM) without significantly affecting the response to saturating (40 nM) vasopressin concentrations (Fig. 1, bottom traces). Pretreatment of the cells with ethanol (100 mM) mimicked the essential features of this inhibition (Fig. 1, middle traces). Ethanol itself causes a transient Ca\(^{2+}\) increase, which we had found earlier to be due to the activation of phospholipase C (1). This response decays over a period of 1–2 min, and the cytosolic Ca\(^{2+}\) concentration returns to resting levels (1, 3). However, the subsequent response to submaximal concentrations of vasopressin (0.1 or 0.4 nM) was inhibited, with respect to both the initial rate of Ca\(^{2+}\) increase and the steady-state Ca\(^{2+}\) concentration attained in those cells (Fig. 1, A and B). Neither of these parameters was affected when...
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**FIG. 5.** Effect of ethanol on agonist-induced inositol phosphate formation in isolated hepatocytes. Isolated cells were labeled with [3H]inositol for 90 min before incubation and extracted and analyzed as described elsewhere (1). The panels on the left show the inositol phosphate production in response to 300 mM ethanol alone. In the other incubations, ethanol (300 mM) was added where indicated, 5 min before the addition of the agonists (1 nM vasopressin, 100 μM phenylephrine, 10 mM NaF + 100 μM AlF₄⁻ and 10 nM glucagon). Samples for extraction and analysis of inositol phosphates by HPLC were taken 30 s and 2 min after these agonists were added. Results are mean ± S.E. from three separate experiments, with individual measurements expressed as the percentage of control samples incubated for the same period without agonists. *p < 0.05; **p < 0.01 comparing incubation with or without ethanol. Mean basal levels/10 mg of cell protein in the absence of stimulants were [3H]Ins(1,4,5)P₃, 237 ± 11 cpm; [3H]Ins(1,3,4)P₃, 196 ± 14 cpm; [3H]InsP₂, 1,063 ± 60 cpm.

saturating vasopressin levels were used (Fig. 1C). In other experiments (not shown), the inhibition of the response to vasopressin was found to persist for as long as ethanol was present in the incubation medium (up to at least 90 min) but reversed slowly, over a period of approximately 60 min, when the cells were washed repeatedly after a short (10-min) treatment with ethanol. In this and all subsequent experiments, 10 mM methylpyrazole was added to prevent secondary effects of ethanol metabolism through alcohol dehydrogenase and to minimize interference by NAD(P)H fluorescence of the Ca²⁺-dependent indo-1 fluorescence.

The concentration dependence of ethanol in inhibiting the rate of Ca²⁺ mobilization at different levels of vasopressin (0.1, 0.4, and 1 nM) is shown in Fig. 2. A significant inhibition (p < 0.05) was observed at all levels of ethanol tested in the range of 6–330 mM, but the sensitivity to ethanol was much more pronounced at the lower doses of vasopressin, approaching the physiologically relevant concentrations of the hormone (which are in the range of 1–100 pM (17)). The inhibition of the rate of Ca²⁺ increase obtained at the highest concentration of ethanol tested was 41.5 ± 4.0% for 1 nM vasopressin, 62.5 ± 4.0% for 0.4 nM vasopressin, and >95% at vasopressin concentrations of 0.1 nM or less. Particularly relevant is the observation that the response to 0.1 nM vasopressin was inhibited by about 30% at 6 mM ethanol, the lowest concentration tested.

A similar inhibition by ethanol was observed with several other agonists that increase cytosolic Ca²⁺ levels by the activation of phospholipase C. As shown in Fig. 3, the intracellular Ca²⁺ mobilization by angiotensin II (4 nM) and EGF (10 nM) was inhibited by the same range of ethanol concentrations which affected the response to vasopressin. The response to these concentrations of agonists was also inhibited by pretreatment of the cells with TPA (10). Also with these agonists, physiologically relevant ethanol concentrations (up to 100 mM) were more effective at physiological agonist concentrations, and the inhibition by ethanol could be overcome with saturating levels of the hormone or growth factor, even at maximally effective ethanol concentrations (data not shown).

In marked contrast, the Ca²⁺ mobilization induced by the α₁-adrenergic agonist phenylephrine (10 μM) was not affected by ethanol pretreatment although the response to this agonist was inhibited by TPA, even at saturating concentrations of the agonist (8, 9). Similarly, the Ca²⁺ mobilization induced by
Effects of Ethanol on Agonist-induced Changes in Inositol Phosphates and Polyphosphoinositides—Receptor-mediated Ca\(^{2+}\) mobilization is secondary to the activation of phospholipase C and the formation of Ins(1,4,5)P\(_3\). Earlier studies (8, 9) had indicated that the target for protein kinase C-mediated inhibition of this system may be the G-protein or the individual receptors that control phospholipase C activity (see also Refs. 18 and 19). We therefore investigated whether ethanol also exerts its effect on the breakdown of polyphosphoinositides and the accumulation of inositol phosphates in response to vasopressin and other agonists. In these experiments, high concentrations of agonists were used in order to maximize the inositol phosphate changes that could be detected. We therefore also employed concentrations of ethanol which were higher than would be observed physiologically in the circulation after ethanol intake.

In the experiment of Fig. 5, isolated hepatocytes were prelabeled with \(^{3}H\)inositol for 90 min, and inositol phosphates were determined in the cell extracts after separation by HPLC as described previously (1). A 30-s exposure of the cells to ethanol (300 mM) increased the levels of Ins(1,4,5)P\(_3\), Ins(1,3,4)P\(_3\), and InsP\(_2\) to 153, 196, and 144%, respectively. However, these changes were transient, and at 2 min after ethanol addition, the levels of these inositol phosphates had almost returned to the basal values, in agreement with our previous data (1, 3). Upon addition of 1 nM vasopressin to untreated cells, the level of Ins(1,4,5)P\(_3\) increased rapidly to 154% of control at 30 s and 194% of control at 2 min. Pretreatment of the hepatocytes with 300 mM ethanol clearly inhibited the accumulation of Ins(1,4,5)P\(_3\). Compared with the addition of vasopressin alone, the increase in the level of Ins(1,4,5)P\(_3\) was inhibited by 50-60% by a 5-min preincubation with ethanol. A parallel inhibition by pretreatment with ethanol was also observed for Ins(1,3,4)P\(_3\) and InsP\(_2\) (Fig. 5, middle and lower panels). This finding indicates that the effect of ethanol was not due to alterations in the further metabolism of the inositol phosphates. In other experiments (not shown) the formation of inositol phosphates in response to EGF (40 nM) and angiotensin II (10 nM) was inhibited similarly by ethanol. Fig. 5 also shows the effect of ethanol pretreatment on inositol phosphate accumulation in response to phenylephrine, glucagon, and fluoride (plus AlCl\(_3\)). Phenylephrine (10 \(\mu\)M) caused an increase in inositol phosphate levels similar to that induced by 1 nM vasopressin. However, in contrast to...
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Fig. 7. Effect of H7 pretreatment on the inhibition by ethanol or TPA of vasopressin-induced [Ca\(^{2+}\)] changes in indo-1-loaded hepatocytes. Experimental conditions were as described for Fig. 1. 100 nM TPA or 100 mM ethanol was added where indicated 2 min before the vasopressin (0.1 nM). In D, E, and F, cells were preincubated for 10 min with 200 μM H7.

the case of vasopressin, pretreatment of the cells with ethanol did not affect the formation of inositol phosphates induced by phenylephrine, in agreement with the experiments on intracellular Ca\(^{2+}\) mobilization. Glucagon (10 nM) caused a much smaller (<20%) increase in inositol phosphates which also was unaffected by pretreatment with ethanol. AlF\(_4\) also activates phospholipase C, presumably by interacting directly with a G-protein (20). Pretreating cells with ethanol slightly enhanced the formation of inositol phosphates in response to fluoride plus AlCl\(_3\) (see also Ref. 21).

Changes in polyphosphoinositide levels were measured in hepatocytes prelabeled with \(^{32}\)P as described previously (1,5). The addition of 300 mM ethanol caused a rapid increase in the levels of PtdIns(4)P and PtdIns(4,5)P\(_2\) to 119 and 118% at 30 s, respectively (Fig. 6). This increase presumably reflects the activation of PtdIns(P) kinases regenerating the substrate for phospholipase C (5). Vasopressin (1 nM) or angiotensin II (10 nM) caused a substantial breakdown of the PtdIns(4)P and PtdIns(4,5)P\(_2\). However, when 300 mM ethanol was added together with these agonists, the decrease in polyphosphoinositide levels was largely inhibited, and with submaximal agonist doses, the net effect was a temporary increase in PtdIns(4)P and PtdIns(4,5)P\(_2\) levels. (Essentially similar results were obtained when cells were preincubated with ethanol prior to vasopressin addition; however, with that protocol, the interpretation of the data is complicated by the different initial levels of polyphosphoinositides in the control and ethanol-treated cells.) In contrast to these results, the decrease in polyphosphoinositide levels upon addition of 10 μM phenylephrine was not affected by ethanol. Glucagon (10 nM) did not cause a significant decrease in polyphosphoinositide levels, and these results were the same when ethanol was present. Thus, not only did ethanol fail to affect the responses to phenylephrine and glucagon, but these agonists also appeared to prevent the PtdIns(P) kinase activation by ethanol.

Effect of Protein Kinase C Inhibitors—In a previous study
Receptor-mediated Phospholipase C Activation in Hepatocytes

**TABLE I**

*Effect of H7 pretreatment on the inhibition by ethanol or TPA of vasopressin-induced inositol phosphate formation*

Experimental procedures were as described for Fig. 5. Where indicated, cells were preincubated for 10 min with 200 μM H7 prior to the addition of 1 nM vasopressin. Ethanol (300 mM) and TPA (100 nM) were added 2 min and 2 min, respectively, prior to vasopressin. Samples for analysis of inositol phosphate by HPLC were taken at 30 s after vasopressin was added. Results are given as the increase in inositol phosphates during the 30-s period after hormone addition, expressed as a percentage of the radioactivity in control samples receiving no additions (mean ± S.E. from three experiments). Mean basal values in cells with no additions were: [3H]Ins(1,4,5)P3, 227 ± 11 and 229 ± 12 cpm/10 mg protein; [3H]Ins(1,3,4)P3, 198 ± 9 and 205 ± 11 cpm/10 mg protein; [3H]InsP2, 1136 ± 60 and 1172 ± 69 cpm/10 mg protein, respectively.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Ins(1,4,5)P3</th>
<th>Ins(1,3,4)P3</th>
<th>InsP2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>+ H7</td>
<td>Untreated</td>
</tr>
<tr>
<td>Vasopressin (1 nM)</td>
<td>63.2 ± 4.9</td>
<td>105.0 ± 8.7</td>
<td>97.6 ± 8.7</td>
</tr>
<tr>
<td>EtoH (300 mM) + vasopressin</td>
<td>34.3 ± 3.7</td>
<td>46.4 ± 5.8</td>
<td>66.3 ± 4.8</td>
</tr>
<tr>
<td>TPA (100 nM) + vasopressin</td>
<td>27.9 ± 3.3</td>
<td>33.7 ± 4.3</td>
<td>87.9 ± 11.2</td>
</tr>
</tbody>
</table>

* p < 0.05, comparing H7-treated and untreated cells.

**TABLE II**

*Effect of H7 pretreatment on the inhibition by ethanol or TPA of vasopressin-induced polyphosphoinositide degradation*

Experimental procedures were as described in Fig. 6. Where indicated, cells were preincubated for 10 min with 200 μM H7 prior to the time of addition of 10 nM vasopressin (time zero), TPA (100 nM) was added 2 min before time zero, and ethanol (300 mM) was added simultaneously with vasopressin. Samples for the analysis of phospholipids were taken just prior to and 30 s after vasopressin or, in incubations that did not receive hormone, at a corresponding time. Results are expressed as a percentage of the radioactivity in the phospholipids at time zero (mean ± S.E. from three separate experiments).

<table>
<thead>
<tr>
<th></th>
<th>PtdIns(4)P</th>
<th>PtdIns(4,5)P2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>+ H7</td>
</tr>
<tr>
<td>None</td>
<td>99.4 ± 2.1</td>
<td>101.4 ± 1.2</td>
</tr>
<tr>
<td>EtoH (300 mM)</td>
<td>117.6 ± 2.5</td>
<td>118.1 ± 2.4</td>
</tr>
<tr>
<td>Vasopressin (10 nM)</td>
<td>75.2 ± 4.7</td>
<td>76.0 ± 3.5</td>
</tr>
<tr>
<td>EtoH + vasopressin</td>
<td>96.8 ± 3.1</td>
<td>81.0 ± 4.1</td>
</tr>
<tr>
<td>TPA (100 nM)</td>
<td>96.3 ± 2.2</td>
<td>100.3 ± 2.1</td>
</tr>
<tr>
<td>TPA + vasopressin</td>
<td>82.7 ± 3.7</td>
<td>77.0 ± 2.9</td>
</tr>
</tbody>
</table>

* p < 0.01, comparing H7-treated and untreated cells.

* p < 0.05, comparing H7-treated and untreated cells.

(3) we reported that the ethanol-induced Ca2+ mobilization was inhibited by phorbol esters that activate protein kinase C. This effect of phorbol esters was prevented by pretreating the cells with the protein kinase C-selective inhibitor H7. H7, as well as some other protein kinase C inhibitors, also enhanced the Ca2+ mobilization by ethanol in cells that were not treated with phorbol esters, indicating that protein kinase C-dependent feedback inhibition played a role in making the response to ethanol transient (3). We therefore investigated whether inhibitors of kinase C also could prevent the inhibitory effect of ethanol on the receptor-mediated phospholipase C activation. Fig. 7 demonstrates the effect of H7 (200 μM) on the Ca2+ mobilization by vasopressin (0.1 nM) in the presence or absence of ethanol (100 mM) or TPA (100 nM). Pretreatment of the hepatocytes with H7, although having no significant effect on the response to vasopressin alone, caused a partial reversal of the inhibitory effects of either of these agents. Staurosporin, another inhibitor of protein kinase C (22), had similar effects (data not shown; the staurosporin effects were measured in fura-2-loaded cells since staurosporin interfered with the fluorescence measurements at the wavelength pair used for indo-1).

Preincubation of hepatocytes with H7 also partially prevented the inhibitory effects of ethanol or TPA on the formation of inositol phosphates (Table I) although it had no effect on the levels of inositol phosphates accumulated in response to the addition of 1 nM vasopressin alone. H7 pretreatment was somewhat less efficient in reversing the inhibition by ethanol than by TPA because it also enhances the phospholipase C activation by ethanol alone (Fig. 7; see also Ref. 3). The effects of H7 pretreatment were reflected equally in the products of inositol phosphate metabolism, Ins(1,3,4)P3 and InsP2, indicating that inhibition of protein kinase C had no significant effect on the conversion of inositol phosphates in the cell under these conditions. The data shown in Table II demonstrate that pretreatment with H7 also prevented the inhibitory effects of ethanol or TPA on the vasopressin-induced degradation of polyphosphoinositides. The changes in polyphosphoinositide levels caused by ethanol or vasopressin alone were not affected by pretreatment with H7. Again, the H7 pretreatment tended to prevent the inhibitory effect of TPA somewhat more effectively than that of ethanol. These experimental results indicate that the inhibition by ethanol of vasopressin-induced phospholipase C activation is at least in part mediated by protein kinase C.

*Synergistic Actions of Ethanol and Phorbol Esters—If*
ethanol exerts its inhibitory effect on receptor-mediated phospholipase C activation exclusively by way of protein kinase C, the effects of phorbol esters and ethanol would be nonadditive. However, the experimental data did not confirm this hypothesis. Fig. 8 shows the effects of a combination of TPA and ethanol on the Ca\(^{2+}\) mobilization induced by 0.4 nM vasopressin. As mentioned above, pretreatment of the hepatocytes with 100 nM TPA or 100 mM ethanol alone partially inhibited the response to vasopressin. Unexpectedly, however, when ethanol (100 mM) was added prior to TPA, the response to vasopressin was almost completely inhibited, indicating that these two agents have a synergistic effect on the receptor-mediated phospholipase C activity. By contrast, when TPA (100 nM) was added prior to ethanol, a treatment that completely inhibited the Ca\(^{2+}\) mobilization by ethanol as reported previously (3), ethanol had no additional effect on the vasopressin-induced Ca\(^{2+}\) mobilization, and the response was essentially the same as when the cells were pretreated with TPA alone. Similar results were obtained when a maximally inhibitory concentration of ethanol (300 mM) was used (data not shown). These data are also supported by the polyphosphoinositide changes shown in Fig. 9, in which cells were preincubated with a maximally inhibitory concentration of TPA (100 nM) for 2 min prior to the addition of ethanol and/or vasopressin. Pretreatment with TPA completely inhibited the ethanol-induced formation of PtdInsP and PtdInsP\(_2\) and partly inhibited the polyphosphoinositide degradation induced by vasopressin (10 nM). When ethanol (300 mM) was added together with vasopressin, the degradation of PtdInsP and PtdInsP\(_2\) was inhibited significantly. However, this partial inhibition by ethanol was completely prevented by pretreating the cells with TPA (100 nM). Similar results were obtained when the inhibition by ethanol of vasopressin-induced inositol phosphate formation was studied. When cells were pretreated with TPA prior to the addition of ethanol and vasopressin, the inhibitory effect of ethanol was prevented; however, when ethanol was added first, followed by TPA, the two agents reinforced each others effect (data not shown). Since TPA can fully inhibit the activation of phospholipase C by ethanol only when it is added at least 1–2 min prior to ethanol (3), this action of ethanol appears to be essential for its subsequent inhibition of receptor-mediated phospholipase C activation. Thus, the effect of ethanol can appropriately be described as a desensitization of the cellular response to hormones. However, the synergistic effects observed when ethanol was added prior to TPA suggest that the
action of ethanol cannot be explained solely by protein kinase C activation.

This synergistic action of ethanol and TPA was studied further in the experiments of Fig. 10, in which the Ca\(^{2+}\) mobilization induced by different concentrations of vasopressin was titrated with TPA in the presence or absence of ethanol. A high concentration of ethanol (300 mM) was used in order to obtain near maximal inhibition of the response to vasopressin (see also Fig. 2). The inhibitory action of the phorbol ester on vasopressin-induced phospholipase C activation decreased markedly with higher hormone concentrations, in agreement with earlier reports (8, 9). The addition of ethanol prior to the phorbol ester enhanced the degree of inhibition at all levels of the hormone. Ethanol had no effect on the EC\(_{50}\) of TPA, indicating that it did not interact directly with protein kinase C-dependent processes. Significantly, the potentiating effect of ethanol was evident even at saturating levels of the phorbol ester, i.e. it enhanced the sensitivity of the vasopressin-induced phospholipase C activation to maximally stimulated protein kinase C.

**Role of Protein Kinase A in the Control of Vasopressin-induced Ca\(^{2+}\) Mobilization**—Earlier experiments by different groups (23, 24) had demonstrated that glucagon and cAMP analogs can potentiate the mobilization of Ca\(^{2+}\) by vasopressin or other phospholipase C-linked agonists. Diamond and co-workers (25, 26) provided evidence that adenylate cyclase activation may occur in different cell types by the release of endogenous agonists such as adenosine. It is conceivable, therefore, that ethanol affects the vasopressin response by indirectly activating the receptor-G protein-phospholipase C complex through protein kinase A. In order to test this hypothesis, we employed the competitive protein kinase A inhibitor, (R_)CAMP-S, which has been used extensively in intact liver cells by Parker-Botelho and co-workers (10, 27-29). Fig. 11 shows the results of a characteristic experiment. Pretreatment of the cells with 100 nM (R_)CAMP-S did not affect the initial rate of the Ca\(^{2+}\) increase induced by 0.4 mM vasopressin although the response was considerably more transient than in the control cells (Fig. 11, A and D). However, (R_)CAMP-S pretreatment markedly enhanced the inhibition of vasopressin-induced Ca\(^{2+}\) mobilization both by ethanol (Fig. 11, B and E) and by TPA (Fig. 11, C and F). The effects of (R_)CAMP-S could be overcome completely by a saturating concentration (2 \(\mu\)M) of the membrane-permeant CAMP analog CPT-cAMP; furthermore, CPT-cAMP diminished the inhibitory effect of ethanol or TPA on vasopressin-induced Ca\(^{2+}\) mobilization (data not shown). The response to higher concentrations of vasopressin (4 nM) was not affected by kinase A inhibition. These findings indicate that the levels of CAMP in the isolated hepatocytes incubated in the presence of ethanol may represent a functionally significant degree of protein kinase A activity, even in the absence of added agonists acting through adenylate cyclase. The addition of aden-
**Effect of (Rp)-cAMP-S on the sensitivity to ethanol and TPA of vasopressin-induced cytosolic [Ca$^{2+}$] increase.** Incubation conditions were as described for Fig. 1. In D, E, and F, cells were preincubated for 5 min with 100 μM (Rp)-cAMP-S prior to the addition of 0.4 nM vasopressin. Traces are from a single experiment representative of three or more experiments showing identical results.

**TABLE III**

*Effect of ethanol and phenylephrine on cAMP accumulation in isolated hepatocytes*

Isolated hepatocytes (6 mg protein/ml) were preincubated for 10 min in the standard incubation medium prior to the addition of ethanol (300 mM) or phenylephrine (10 μM). Samples were taken after 30 s and analyzed for cAMP as described in the Experimental section. Results are means ± S.E. (basal levels 394 ± 22 fmol/mg protein) in three separate experiments.

<table>
<thead>
<tr>
<th>Additions</th>
<th>[cAMP] Increase/30 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>368 ± 13</td>
</tr>
<tr>
<td>Ethanol (300 mM)</td>
<td>447 ± 12</td>
</tr>
<tr>
<td>Phenylephrine (10 μM)</td>
<td>383 ± 15</td>
</tr>
<tr>
<td>Phenylephrine + ethanol</td>
<td>425 ± 12</td>
</tr>
</tbody>
</table>

* $p < 0.01$ compared with no additions.

Glucagon is a potent adenylate cyclase-activating hormone in liver cells. Phenylephrine acts primarily on α1-adrenergic receptors to activate phospholipase C but can, under certain conditions, increase cellular levels of cAMP through the same receptors (30). Table III shows that cAMP accumulation in isolated hepatocytes was enhanced by about 20% in response to phenylephrine (10 μM), in the presence or absence of ethanol (300 mM). As shown in Fig. 12, pretreatment of the cells with (Rp)-cAMP-S sensitized the phenylephrine-induced Ca$^{2+}$ mobilization by ethanol or the inhibition by ethanol of the vasopressin-induced increase in cytosolic Ca$^{2+}$ (data not shown). Thus, adenosine release by the cells did not play a role in the responses studied here.
zation to ethanol. The initial rate of Ca^{2+} increase induced by a low concentration of the agonist (4 μM) was inhibited by ethanol in cells that were pretreated with (Rp)-cAMP-S (54 ± 3% inhibition by 300 mM ethanol in four separate experiments; p < 0.01) but not in untreated cells. In the absence of ethanol, the protein kinase A inhibitor made the phenylephrine-induced increase in cytosolic [Ca^{2+}] more transient, similar to its effect on the Ca^{2+} mobilization induced by vasopressin.

DISCUSSION

In previous studies (1, 3, 13, 31), we demonstrated that the activation of polyphosphoinositide-specific phospholipase C by ethanol in isolated hepatocytes is transient due to inhibition of the enzyme complex by protein kinase C, which is activated by the diacylglycerol formed in the reaction. This feedback inhibition of phospholipase C after activation by ethanol may be characterized as homologous desensitization, mediated by protein kinase C; after pretreatment with submaximal concentration of ethanol, the enzyme complex still responds to subsequent stimulation with a higher concentration of ethanol (1, 31). The experiments reported here demonstrate that ethanol treatment also inhibits the response to hormones that activate phospholipase C through a receptor-mediated mechanism. This inhibitory effect of ethanol has the characteristics of heterologous desensitization. First, activation of phospholipase C is required for ethanol to inhibit the subsequent response to hormones; when the cells are pretreated with TPA, a condition that completely eliminates the phospholipase C activation by ethanol, there is no additional effect of ethanol on the response to vasopressin. In contrast, when ethanol is added first to activate phospholipase C prior to TPA addition, the effects of these two agents are synergistic. Second, the effect of ethanol is observed with several different agonists that activate phospholipase C through a receptor-mediated mechanism. Third, ethanol de
creases the sensitivity to these agonists, but its effects can be overcome by saturating agonist concentrations. Fourth, in common with patterns of heterologous desensitization displayed in other systems, the sensitivity to hormones is recovered only gradually after removal of ethanol, indicating that distinct processes control the onset of the inhibition and its reversal.

The data presented here indicate that at least part of the desensitization by ethanol is due to the activation of protein kinase C. Pretreating the cell with an inhibitor of protein kinase C (H7, staurosporin) partly prevented the inhibitory action of ethanol even though it enhanced the ethanol-induced activation of phospholipase C. Furthermore, the characteristics of the desensitization by ethanol paralleled in many respects the inhibition by phorbol esters of receptor-mediated phospholipase C activation (8-12). Similar to TPA, the effects of ethanol were exerted at or prior to phospholipase C, presumably at the level of the receptor or the G-protein which regulates phospholipase C activity, and both agents were most potent at low agonist concentrations. Furthermore, (R)-cAMP-S, an inhibitor of protein kinase A, potentiated the inhibition of vasopressin-induced Ca\(^{2+}\) mobilization both by ethanol and by TPA. By contrast, activation of protein kinase A appears to counteract the effects of both ethanol and phorbol esters. These experiments also indicate that ethanol does not act in this system by activation of adenylate cyclase, in contrast to several other cell types (25, 26, 32).

However, our initial hypothesis (3, 13) that ethanol acts through protein kinase C did not reflect adequately the range of interactions of ethanol with the phospholipase C-mediated signaling processes in the liver cell. In particular, the observation that the effects of ethanol are exerted even in the presence of a saturating TPA concentration (Fig. 10) is evidence for an additional site of action of ethanol.

The data provide some evidence that this additional site of action could be at the receptor level. First, the sensitivity of the cells to ethanol appeared to be lower at higher levels of vasopressin (Fig. 2), with an EC\(_{50}\) of about 10 mM at 0.1 nM hormone, increasing to >100 mM at 1 nM vasopressin. Since physiological levels of vasopressin are in the range of 1–100 pm (17), the high sensitivity to ethanol may well be physiologically relevant. By contrast, the sensitivity to TPA pretreatment was not affected by the hormone concentration; as illustrated in Fig. 10, the EC\(_{50}\) for TPA was 5–10 nM, irrespective of the vasopressin concentration, and maximal inhibition was obtained at 100 nM TPA or higher. The competitive relationship between the hormone and ethanol suggests that ethanol may act at a site prior to the target of the TPA's action on the signal transduction pathway.

A second argument in support of an action of ethanol at the receptor level is the target specificity of its inhibitory effect; we found a complete absence of any ethanol effects on phospholipase C activation through \(\alpha_1\)-adrenergic receptors or glucagon under standard conditions. However, the interpretation of these findings is not altogether straightforward. In the first place, the mechanism by which glucagon activates phospholipase C is not established unequivocally. Although Wakelam et al. (33) have provided evidence in support of a distinct glucagon receptor coupled to phospholipase C, other data (34) indicate that activation of protein kinase A by cAMP may be sufficient to stimulate phospholipase C. Hence, the activation of phospholipase C by glucagon may be indirect and may not involve a receptor that is coupled directly to that effector system. Second, activation of protein kinase A by cAMP analogs could overcome the inhibition of vasopressin-induced Ca\(^{2+}\) mobilization by ethanol whereas the protein kinase A inhibitor (R)-cAMP-S potentiated the inhibitory effect of ethanol. Under the conditions used, not only glucagon but also phenylephrine caused a detectable increase in cAMP levels which appeared to be sufficient to overcome an effect of ethanol. This is indicated by the finding that phenylephrine-induced Ca\(^{2+}\) release was sensitized to inhibition by ethanol by pretreating the cells with (R)-cAMP-S (Fig. 12). Thus, protein kinase A activation may modulate the sensitivity to ethanol of both the vasopressin-inhibited and the phenylephrine-induced Ca\(^{2+}\) increase, but the fact that phenylephrine caused a significant increase in cAMP levels would make it more resistant to ethanol. In contrast to vasopressin-induced responses, the sensitivity of phenylephrine-induced phospholipase C activation to TPA was not affected by protein kinase A activation (data not shown). Hence, the site of action of phorbol ester-activated protein kinase C on the signaling mechanisms of this agonist does not appear to be shared by ethanol pretreatment. Earlier studies by Exton and co-workers (8) have indicated that the \(\alpha_1\)-adrenergic signaling system differs from that activated by vasopressin in that the \(\alpha_1\)-adrenergic receptor itself may be a target for protein kinase C. It is conceivable that the targets for protein kinase C activated with internally generated diacylglycerol may be different from those with externally added phorbol esters. Alternatively, protein kinase A activation may affect the sensitivity of protein kinase C to diacylglycerol or the accumulation of diacylglycerol in response to ethanol.

An analysis of the polyphosphoinositide data of Fig. 6 indicates that both glucagon and phenylephrine prevented the activation of PtdIns(3) kinase by ethanol. The mechanism(s) that control PtdIns(3) kinases are not well understood, and it is not clear why ethanol causes a substantial increase in the levels of polyphosphoinositides as opposed to receptor-mediated agonists, which uniformly decreased the level of these compounds. The data of Fig. 5 suggest that glucagon and phenylephrine, agonists that increased cAMP levels in the cells, selectively inhibited the PtdIns(3) kinase activation by ethanol. Since cAMP analogs enhance the ethanol-induced Ca\(^{2+}\) mobilization, the activation of the PtdIns(3) kinases would thereby be dissociated from the phospholipase C activation by ethanol. It should be pointed out that the isomeric composition of the polyphosphoinositides formed in response to ethanol treatment has not yet been determined; it is possible that ethanol stimulates specifically the PtdIns-3 kinase, which does not produce a precursor for Ins(1,4,5)P\(_3\) formation but which may act to modulate phospholipase C activation by competition for its substrate (35).

Taken together, the data presented in this paper can be interpreted within a model in which protein kinase A and protein kinase C have inverse effects on the agonist-induced Ca\(^{2+}\) mobilization in hepatocytes, with protein kinase C inhibiting the response and protein kinase A decreasing the sensitivity of the system to inhibition by protein kinase C. Ethanol interferes with this balance not only by generating diacylglycerol which can activate protein kinase C, but also by shifting the balance of the system, making the receptor-mediated phospholipase C activation more sensitive to the actions of protein kinase C. The mechanism by which ethanol induces this shift in sensitivity remains to be determined. The observation that pretreatment of the cells with TPA can prevent this effect indicates that the mere presence of ethanol in the system is not sufficient to induce this shift in sensitivity. Moreover, when ethanol was removed from the cells by repeated washing, the inhibition of vasopressin-induced Ca\(^{2+}\) mobilization remained in effect and recovered only gradually over a period of 60 min. It may be that ethanol affects the
function of other protein kinases or phosphatases in the cell which influence the susceptibility of the receptor-coupled phospholipase C reaction to protein kinase C, by covalent modification of a critical component of the signaling system, thereby affecting receptor-G protein coupling. It is also conceivable that ethanol affects the recently identified phospholipase D activity that can be stimulated by a receptor-mediated agonist such as vasopressin or by protein kinase C (36, 37). Ethanol can interact with this process by serving as an alternative substrate, leading to formation of phosphatidyl-ethanol (37). The role of these reactions in the balance of phospholipid-dependent signal transduction processes in liver is currently under investigation.

Physiologically, an interference of ethanol with the cellular signal transduction processes could have important implications for the control of cellular metabolism, both short and long term. In the majority of experiments shown in this paper, unphysiologically high concentrations of vasopressin and other agonists were used in order to improve the quantitative analysis by maximizing the activation of the signaling processes in the cell. Relatively high concentrations of ethanol (100–300 mM) were used to give readily detectable effects under those conditions. However, as shown in the experiments of Figs. 1–3, at lower, more physiologic hormone concentrations, the cells were very sensitive to ethanol concentrations that were well within the physiologically relevant range. Specifically, we found that the response of liver cells to vasopressin concentrations in the upper end of the physiologically relevant range (10–100 pM) were inhibited 50% or more by 5–10 mM ethanol. These concentrations of ethanol can be attained in the circulation, even after moderate drinking. Our data indicate that a short peak of ethanol exposure can induce a desensitization that may persist even after the ethanol concentration has declined. These characteristics raise the potential for a significant prolonged effect of ethanol exposure on cellular control mechanisms which may be relevant for the adaptive changes seen in response to chronic alcohol intake (38).

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REFERENCES