Ethanol-induced Mobilization of Calcium by Activation of Phosphoinositide-specific Phospholipase C in Intact Hepatocytes

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Ethanol interacts with biological membranes and affects their physical and chemical properties (see Ref. 1 for a review). The consequences of these interactions for cellular functions have not yet been well defined. A variety of cells responds to the continued presence of ethanol by inducing structural changes in their membranes so as to make them more resistant to the physical effects of ethanol. Both the acute effects of ethanol and the adaptations occurring in cells chronically exposed to ethanol can influence the activity of membrane-bound enzymes, including ion transport systems (1).

In an earlier report (2), we demonstrated that the Ca²⁺ pump activity in microsomal preparations from rat liver is affected by ethanol treatment. Other studies (3-5) reported an effect of ethanol on the activity of the Ca²⁺-ATPase in brain and erythrocyte membranes and on voltage-operated Ca²⁺ channels in neurons. These observations suggest the possibility that ethanol interferes with normal mechanisms of calcium homeostasis in liver and other tissues.

Calcium serves an important function as an intracellular messenger (6). The steady state level of cytosolic free Ca²⁺ in liver cells is maintained in the range of 0.1-0.2 μM (6-9) by the action of calcium transport systems in the plasma membrane, endoplasmic reticulum, and mitochondria, and by binding to intracellular constituents. Certain hormones and other agonists (α₂-adrenergic agonists, vasopressin, angiotensin II, ATP) can increase cytosolic calcium levels by mobilizing intracellular calcium stores (6-10). A primary step in the action of such agonists is the activation of a phosphoinositide-specific phospholipase C in the plasma membrane, resulting in the intracellular release of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (see Refs. 11-13 for reviews). Ins(1,4,5)P₃ causes the release of calcium from specific nonmitochondrial pools (14). The resultant increase in cytosolic free Ca²⁺ activates Ca²⁺-dependent protein kinases and other Ca²⁺-dependent enzymes (11-13). Diacylglycerol, in the presence of basal levels of calcium, activates protein kinase C (15), which can phosphorylate and presumably regulate the function of several proteins (12); most of these proteins have not yet been functionally characterized. Thus, receptor-mediated activation of phospholipase C leads to a complex network of intracellular signals resulting in an increased phosphorylation of various soluble and membrane-bound proteins (16).

In our studies of the effects of ethanol on calcium homeostasis in isolated hepatocytes, we first observed a marked activation of glycogen phosphorylase. The activation of phosphorylase kinase, detected by the conversion of phosphorylase a to phosphorylase b, has been used extensively as an indicator...
of cytosolic calcium levels in liver (6). However, activation of phosphorylase kinase in liver and other tissues can also be induced by several other mechanisms. The enzyme can be activated by phosphorylation, e.g., by cAMP-dependent protein kinase (17, 18). The enzyme from skeletal muscle is also activated by phosphorylation, induced by CAMP-dependent protein kinase (17, 18), by limited proteolysis (19), and by high concentrations (>0.3 M) of organic solvents, including ethanol (20). The effect of these agents on liver phosphorylase kinase has not been studied in detail, but the responses of the enzyme from liver and skeletal muscle differ in several respects (21, 22). In this report we investigate the mechanism of ethanol-induced phosphorylase activation and its relationship to calcium homeostasis and signal transduction pathways in the liver cell. The results demonstrate that ethanol causes a mobilization of calcium from intracellular hormone-sensitive stores by activation of the phosphoinositide-specific phospholipase C.

**EXPERIMENTAL PROCEDURES**

**Preparations and Incubation Conditions**

Isolated hepatocytes were prepared from male Sprague-Dawley rats, starved overnight. The collagenase perfusion method of Berry and Prentki (23) was described by Meyers et al. (24). The washed cell suspension (30-50 mg of protein/ml) was kept at room temperature until use. Viability of the cells was more than 90%, as measured by trypan blue exclusion.

Incubations were carried out in 25-ml flasks in a shaking water bath at 37°C in Krebs-Ringer bicarbonate buffer, containing 2% bovine serum albumin and either 10 mM lactate plus 1 mM pyruvate or 15 mM glucose. The pH was 7.4 and the gas phase contained 95% O2, 5% CO2. The incubations contained 3-5 mg of cell protein/ml (1.8-2.8 × 10⁶ cells/ml) in a final volume of 5 ml. For the determination of phosphorylase a activity, hepatocytes were preincubated for 20-30 min to obtain a stable basal activity. Reactions were stopped by rapidly adding a 0.1-ml sample of the incubation medium into 0.5 ml of ice-cold stopping medium containing 50 mM NaF, 25 mM β-glycerophosphate, 10 mM MES, 2 mM EDTA, and 0.2 mM digitonin or by transferring 0.1 ml of the cell suspension into a test tube containing liquid N2O, followed by 0.3 ml of the stopping medium. Samples for phosphorylase kinase activity were stopped by the same procedure, except that EDTA was omitted from the stopping medium.

For isotopic labeling, the cells were preincubated for 90 min in medium containing either 10 μCi/ml [3H]inositol and 10 mg of cell protein/ml or 50 μCi/ml [32P]orthophosphate and 10 mg of cell protein/ml. For the measurement of [3H]-labeled phosphorylids, 0.15 ml of cell suspension was quenched with 0.56 ml of chloroform/methanol/HCl (200:100:5). [3H]Inositol phosphates were extracted from cells prelabeled with [3H]inositol by quenching 0.6 ml of cell suspension into 0.2 ml of 14% perchloric acid. Quin2-loaded cells were prepared essentially as described by Tsien et al. (25, 26) and by Charest et al. (9) by incubating the hepatocytes (6-8 mg of protein/ml) for 20-30 min in a medium containing 120 mM NaCl, 20 mM Hepes, 1.2 mM MgCl₂, 1.0 mM CaCl₂, 0.1 mM EDTA, 1.2 mM potassium phosphate, 4.8 mM KCl, 0.1% Ficoll with 80 μM quin2-tetracycloxyethylene (quin2-AM). Quin2 fluorescence was measured with a MFP-44B Perkin-Elmer spectrophotometer (ratio mode) at an excitation wavelength of 339 nm (slit width, 10 nm) and an emission wavelength of 492 nm (slit width, 20 nm). The temperature was maintained at 37°C, and the cells were kept in suspension in the cuvette by gentle stirring. Maximum fluorescence levels were obtained by permeabilizing the cells with a minimal amount of digitonin (2-3 nmol/mg of protein). In many incubations a gradual oxidation of cellular NAD(P)H was initiated by this digitonin treatment, causing a drift in the fluorescence which interfered with the estimation of the maximal and minimal quin2 fluorescence levels. Therefore, before estimating the total quin2-related fluorescence, by the addition of EGTA/Tris (final pH 8.5) (26) an excess of digitonin (50 nmol/mg of protein) was added, causing a rapid and complete oxidation of NAD(P)H. Corrections for extracellular quin2 (26) were obtained by using EGTA/Tris to the cuvette without cells. Digitonin, and the total quin2 content was obtained after back titration with CaCl₂. Concentrations of intracellular quin2 were in the range of 0.5-0.8 mM. The concentration of cytosolic free calcium was calculated assuming a Kₐ for the quin2-calcium complex of 115 nM as described by Tsien et al. (26), after correction for extracellular quin2.

For measurement of Ca²⁺ efflux using arsenazo III, cells (5-6 mg of protein/ml) were incubated in a Ca²⁺-free modified Hanks’ medium (7, 8) containing 20 μM arsenazo III. The calcium-dependent change in absorbance was measured in an Aminco DW22 spectrophotometer at a wavelength pair of 570/540 nm. Calcium was determined by the addition of small quantities of a standard calcium solution.

**Asays**

**Phosphoinositides**—For the analysis of the [3P]-labeled lipids, 0.188 ml of 2 M KCl, 10 mM EDTA and 0.188 ml of chloroform/methanol were added to the chloroform/methanol/HC1 extract. The lower chloroform layer was taken and evaporated in a Rotovac evaporator. The samples were resuspended in chloroform/methanol (2:1) and separated on octadecyl-treated Silica Gel 60 plastic-backed thin layer chromatography plates (20 × 20) (EM Reagents) using a solvent system composed of chloroform/aceton/methanol/acetic acid/H₂O (160:60:52:48:32) (28). [3P]-Labeled lipids were located by autoradiography using X-Omat AR X-ray film (Kodak) and identified by using lipid standards stained with 1% vapor. The radioactivity in each spot was quantitated by cutting out the indicated areas and liquid scintillation counting in H2O (Cerenkov counting).

**Inositol Phosphates**—Perchloric acid extracts of [3H]-inositol-labeled hepatocytes were centrifuged to remove precipitated protein and lipid and neutralized on ice with KOH to pH 7.0. The extract was stored frozen for up to 3 days prior to separation of inositol phosphates by HPLC. The HPLC procedure was based on that described by Irvine et al. (29) for the separation of Ins(1,4,5)P₃ from Ins(1,3,4,5)P₄ with modifications similar to those described by Dzatti et al. (30) to elute InP₃. Briefly, the sample was loaded onto a 250 × 4.6-mm Partisil SAX-10 column in 0.8 ml and washed through with H₂O (0-7 min of elution). A constant flow rate of 1.6 ml/min was employed throughout the separation. Subsequently, a linear gradient from H₂O to 0.8 M HCOONH₄ (pH adjusted to 7.7 with H₃PO₄) was run at a rate of 35 mM HCOONH₄/min (7-30 min of elution), at which point the gradient was reduced to 5 mM HCOONH₄/min. This slow gradient was continued over the elution period (30-38 min) during which time ATP, Ins(1,3,4)P₃, and Ins(1,4,5)P₃ were eluted from the column. Finally, the gradient was increased to a rate of 150 mM HCOONH₄/min and run to a maximum value of 2.2 M HCOONH₄, where it was held constant for 4 min (38-51 min of elution). Fractions were collected at 2-min intervals over the period 7-30 min, at 0.4-min intervals from 30-38 min, and at 1-min intervals from 38-51 min. The "H content of these fractions was determined by liquid scintillation counting in 2 ml of scintillation fluid (Digital scintillation liquid, National Diagnostics). Carrier-free [32P]orthophosphoric acid and 50-[2-3H]inositol (15 Ci/mmol in 95% ethanol) was obtained from American Radiolabeled Chemicals. Immediately prior to use of the [3H]inositol, the ethanol was removed by evaporation and the isotope redissolved in water. To remove any impurities which might affect scintillation counting, the [3H]inositol solution was treated with a small quantity of Dowex 1-X8 anion-exchange.

**Materials**

Carrier-free [3P]orthophosphoric acid and 50-[2-3H]inositol (14.3-triphosphate (1 C/mmol) were purchased from American Corp. [3H]inositol (15 Ci/mmol in 95% ethanol) was obtained from American Radiolabeled Chemicals. Immediately prior to use of the [3H]inositol, the ethanol was removed by evaporation and the isotope redissolved in water. To remove any impurities which might affect scintillation counting, the [3H]inositol solution was treated with a small quantity of Dowex 1-X8 anion-exchange.

1 M. Prentki, personal communication.
resin (formate form), 200-400 mesh (Bio-Rad). Ionophore A23187 was obtained from Behring Diagnostics. Arginine vasopressin was obtained from Vega Biochemicals. Lipid standards, quin2, quin2-AM, and collagenase were purchased from Sigma. Other chemicals and biochemicals of the highest purity commercially available were purchased from Sigma or from Fisher.

RESULTS

Characteristics of Ethanol-induced Phosphorylase Activation—The addition of ethanol (100 mM) to a suspension of isolated hepatocytes caused a rapid conversion of glycogen phosphorylase from its inactive (b) form to the active (a) form, indicating an activation of phosphorylase kinase. The phosphorylase a activity peaked approximately 30 s after the addition of ethanol and declined gradually over a period of 5-10 min (Fig. 1). This kinetic pattern of phosphorylase activation by ethanol was comparable to that brought about by calcium-mobilizing hormones such as vasopressin or α1-adrenergic agonists (7-9, 34), but the subsequent decline in activity was somewhat faster after ethanol addition. Fig. 2 shows the concentration dependence of phosphorylase activation by ethanol after a 30-s exposure. Significant (p < 0.05)

![Image of Fig. 1: Time course of phosphorylase activation by ethanol in intact hepatocytes.](image1)

**Fig. 1.** Time course of phosphorylase activation by ethanol in intact hepatocytes. Hepatocytes (5 mg of protein/ml) were preincubated for 30 min before the addition of 100 mM ethanol. Reactions were stopped by addition of a sample of the cell suspension to liquid N2. Phosphorylase a was assayed as described under “Experimental Procedures.” Results are mean ± S.E. for 4-7 different experiments.

![Image of Fig. 2: Effect of ethanol concentration on phosphorylase activation.](image2)

**Fig. 2.** Effect of ethanol concentration on phosphorylase activation in intact hepatocytes. Reaction conditions were as described in the legend to Fig. 1. Reactions were stopped 30 s after the addition of ethanol by the addition of stopping medium (see “Experimental Procedures”). Results are mean ± S.E. for 5 individual experiments.

differences from control incubations were obtained with ethanol concentrations of 25 mM and higher, and maximal activation of phosphorylase (80-90% of total phosphorylase activity) was found above 200 mM ethanol. Ethanol in this concentration range had no effect on phosphorylase activity when added after disruption of the cells; thus, the activation of phosphorylase was not due to a direct activation of the enzyme by ethanol. Phosphorylase kinase activity, measured in broken cell preparations with commercial muscle phosphorylase b as substrate, was consistently activated by ethanol at concentrations of 500 mM or more, but ethanol had no effect at concentrations up to 200 mM (data not shown). These observations are in agreement with the data reported by Singh and Wang (20) on purified muscle phosphorylase kinase. These authors found substantial activation of phosphorylase kinase by ethanol at concentrations in excess of 1 M but little effect below 300 mM ethanol. In other control experiments, ethanol in this concentration range had no significant effect on the cellular level of cAMP (0.37 ± 0.05 and 0.43 ± 0.07 pmol/mg of protein before and 30 s after 200 mM ethanol, respectively, in three separate experiments [see also Ref. 35]). The activation of phosphorylase was not affected by 4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase (data not shown). After the phosphorylase response to the addition of 100 mM ethanol had decayed to basal levels, a similar transient activation of phosphorylase could be induced by a second addition of 100 mM ethanol (Fig. 3). This repeated response occurred even in the presence of 4-methylpyrazole and despite the fact that the ethanol concentration had not substantially decreased during the 15-min incubation time.

3 Ethanol oxidation, which occurs in isolated hepatocytes at a rate of approximately 10 nmol/min/mg of protein at 37 °C (36) and which is at least 80% inhibited by 4-methylpyrazole (37), can account for the disappearance of less than 0.2% of the ethanol added in the experiment of Fig. 3.
Thus, neither the onset of phosphorylase activation nor its decay is dependent on the oxidation of ethanol by alcohol dehydrogenase. When 200 mM ethanol was added, either at zero time or after incubating the cells under control conditions for 15 min, the response of phosphorylase was slightly higher and its decay slower than with the 100 mM ethanol additions, even though the final concentration of the agent was the same.

The activation of phosphorylase was not specific for ethanol; other organic solvents had similar effects (Table I). In a series of short-chain n-alcohols, the degree of phosphorylase activation increased with chain length. This finding suggests that the effect is a function of the hydrophobic properties of the solvent. The general anesthetic halothane, which shares many of the physical effects of ethanol on membranes due to its hydrophobic nature (1), also increased phosphorylase activity in intact hepatocytes. Solvents with higher dielectric constants than the straight-chain alcohols (dimethyl sulfoxide, dimethylformamide) tended to have a qualitatively similar, but less pronounced effect in activating phosphorylase. In the concentrations used here (<2%, v/v), the activation of phosphorylase was not specific for ethanol.

Role of Calcium in Phosphorylase Kinase Activation—The contribution of changes in cytosolic calcium to the ethanol-induced phosphorylase kinase activation was tested in hepatocytes loaded with the calcium indicator quin2 (25, 26). At the fluorescence wavelength pair used for measuring the calcium-quin2 complex (339 and 492 nm), there is a marked contribution of NAD(P)H fluorescence to the signal. Since ethanol addition causes extensive reduction of NAD(P)H in hepatocytes, the contribution of quin2-related fluorescence has to be separated from NAD(P)H fluorescence. In Fig. 4, fluorescence changes in quin2-loaded cells (a–d) are compared with those observed in unloaded cells (e–h). In incubation a and e the hepatocyte suspension was pretreated with a low concentration (7 mM) of ethanol. This concentration is well above the $K_m$ of alcohol dehydrogenase for ethanol (37) and gives a maximal reduction of NAD(P)H but has little effect on phosphorylase activity (see Fig. 2). A subsequent addition of 300 mM ethanol to unloaded cells (Fig. 4e) induced a partial reoxidation of NAD(P)H, presumably because of substrate inhibition of alcohol dehydrogenase (38). In quin2-loaded cells (Fig. 4a) the same concentration of ethanol caused a rapid transient increase in fluorescence superimposed on the decrease in NAD(P)H fluorescence. After permeabilization with a low level of digitonin, quin2 fluorescence was maximized, while a downward drift was found in the fluorescence of unloaded cells; addition of a high level of digitonin caused a rapid oxidation of NAD(P)H of similar magnitude in both incubations. Ethanol-induced changes in NAD(P)H fluorescence can largely be prevented by pretreatment of the cells with 4-methylpyrazole (15 mM) (Fig. 4, b and f); high concentrations of ethanol now caused a minor increase in NAD(P)H level (Fig. 4f). In quin2-loaded cells, addition of 4-methylpyrazole gave a small transient increase in fluorescence which decayed to base line after 2–3 min. Ethanol (300 mM) again

| TABLE I |
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| Effect of organic solvents on phosphorylase activation in intact hepatocytes |

Reaction conditions were as described in the legend to Fig. 1. Phosphorylase activity was determined 1 min after the addition of the different solvents. Results are means ± S.E. of the number of experiments shown in parentheses. Statistical significance: $p < 0.05$ compared to control for all conditions.

| Addition      | Concentration | Phosphorylase a |
|---------------|---------------|-----------------|
| None          | mM            | nmol/min/mg protein |
| Methanol      | 50           | 60.4 ± 6.5 (5)  |
| Ethanol       | 50           | 71.1 ± 14.6 (5) |
| 1-Propanol    | 50           | 201.2 ± 96.2 (5) |
| 1-Butanol     | 50           | 236.7 ± 74.2 (5) |
| Dimethyl sulfoxide | 50           | 61.1 ± 14.1 (5)  |
| Dimethylformamide | 50      | 59.7 ± 10.5 (4)  |
| Tetrahydrofuran | 50          | 145.9 ± 22.8 (4) |
| Halothane     | 10           | 99.7 ± 7.9 (3)  |
Ca2+ responsiveness in the cytosol. In Table I, the effect of varying quin2 was not consistently affected by ethanol treatment, although with phenylephrine (2 μM), the peak of the calcium response occurred due to cell damage induced by ethanol addition. The steady state free cytosolic Ca2+ level measured 3 min after the addition of the stimulus was relatively unaffected by the quin2 loading.

**Intracellular Origin of Calcium Mobilized by Ethanol**—The ethanol-induced change in cytosolic Ca2+ level did not require the presence of calcium in the incubation medium. As shown in Fig. 5A, the addition of excess EGTA immediately before ethanol had no effect on the time course of phosphorylase activation and its decay. Similarly, the fluorescence response in quin2-loaded cells was not affected by the addition of excess EGTA (not shown). Preincubation of the cells for 30 min in a calcium-free medium with 1 mM EGTA decreased the ethanol-induced increase in phosphorylase activity (Fig. 5B); presumably this is due to the depletion of calcium from intracellular pools over time. Similar effects of pretreatment with 1 mM EGTA were observed on phenylephrine-induced activation of phosphorylase; however, in contrast to ethanol, the decay of the phenylephrine-induced calcium mobilization was much faster in the absence of extracellular calcium than in the standard calcium-containing medium (35).

In further experiments the hepatocytes were washed in a calcium-free medium and incubated in a buffer containing the calcium indicator, arsenazo III (Fig. 6). Under these conditions, different calcium-mobilizing agents (A23187, phenylephrine, vasopressin) caused a rapid efflux of calcium from the cells, in agreement with the intracellular origin of the calcium mobilized by these agents. As shown in Fig. 6, ethanol (300 mM) similarly caused a small degree of calcium efflux from the cells and decreased the A23187-mobilizable Ca2+ pools.

**Relationship with Hormone-induced Calcium Mobilization**—In liver cells, vasopressin and α1-adrenergic agonists mobilize calcium from an intracellular storage site, presumably located in the endoplasmic reticulum (14, 39). In the experiment of Fig. 7, we investigated the relationship between the calcium pools affected by ethanol and by these hormones in quin2-loaded cells. The addition of phenylephrine (2·10⁻⁵ M) to

| Stimulus       | Quin2 loading | Cytosolic free Ca²⁺ | Steady state |
|----------------|---------------|---------------------|--------------|
| Ethanol (300 mM) | 0.6           | 183 ± 13            | 460 ± 47     | 207 ± 21     |
|                | 1.5           | 148 ± 27            | 283 ± 55     | 156 ± 37     |
|                | 3.1           | 124 ± 17            | 150 ± 18     | 161 ± 19     |
| Phenylephrine (2 μM) | 0.6           | 164 ± 3             | 400 ± 21     | 194 ± 19     |
|                | 1.5           | 112 ± 12            | 256 ± 5      | 139 ± 5      |
|                | 2.9           | 106 ± 15            | 207 ± 34     | 166 ± 28     |

**Fig. 5. Effect of EGTA on ethanol-induced phosphorylase activation.** A, after 30 min of preincubation in a standard medium containing 1.2 mM CaCl₂, cells were challenged with 100 mM ethanol at t = 0; O, EGTA (2.5 mM) was added 30 s before ethanol. B, ○, cells were preincubated in a calcium-free medium in the presence of 1 mM EGTA; ●, control incubations in the presence of 1.2 mM CaCl₂.
Calcium Mobilization and Phospholipase C Activation by Ethanol

**FIG. 6.** Cellular calcium efflux induced by ethanol, phenylephrine, and A23187. Cells (5 mg of protein/ml) were incubated in a calcium-free modified Hanks' medium containing 20 μM arsenazo III. Calcium efflux (upward deflection) was measured in an Aminco DW2a dual wavelength spectrophotometer (375–385 nm). Phenylephrine was added to a concentration of 10⁻⁸ M. A23187 was added as a solution in dimethyl sulfoxide, to a final concentration of 20 μM.

**FIG. 7.** Interrelationship between ethanol-induced and phenylephrine-induced calcium mobilization in quin2-loaded hepatocytes. The scale of cytosolic calcium concentration was calculated in each incubation from the maximal (digitonin-treated) and minimal (digitonin + EGTA-treated) fluorescence levels.

hepatocytes pretreated with 4-methylpyrazole induced a Ca²⁺ mobilization response with characteristics comparable to those reported by others (e.g. Refs. 9 and 40). In the presence of extracellular calcium, a transient peak (up to 1 μM cytosolic Ca²⁺) was followed by a gradual decline to a new steady state of about 0.3 μM free Ca²⁺. When cells were pretreated with ethanol (250 mM), the fluorescence response to hormone addition was qualitatively similar and the peak calcium level was comparable to that found in cells that had not received ethanol (Fig. 7). By contrast, pretreatment of the cells with phenylephrine completely prevented the calcium mobilization in response to 250 mM ethanol. This observation is confirmed by the Ca²⁺ efflux experiment shown in Fig. 6; pretreatment of the cells with phenylephrine inhibited the ethanol-induced efflux of calcium. Thus, the ethanol-induced calcium mobilization appears to be completely dependent on the presence of calcium in the hormone-sensitive calcium pool in the cell. Similar results were obtained when calcium mobilization was measured by phosphorylase activation (data not shown). The ethanol-induced response could also be prevented by pretreating the cells with a saturating concentration of vasopressin (35).

Phenylephrine-induced calcium mobilization in hepatocytes is mediated by Ins(1,4,5)P₃, and the question arises whether ethanol acts directly on the Ins(1,4,5)P₃-sensitive calcium stores. In hepatocytes in which the plasma membrane has been made permeable by treatment with digitonin, the hormone-sensitive calcium pools can be mobilized directly by the addition of Ins(1,4,5)P₃ (14). In the experiment of Fig. 8 the cells were preincubated in the presence of digitonin and ATP and allowed to reach a steady state free Ca²⁺ concentration (approximately 200 nM). The calcium level was measured by the fluorescence of quin2 which was included in the medium to both monitor and buffer the free Ca²⁺ concentration. Under these conditions, addition of Ins(1,4,5)P₃ (1 μM) caused a rapid release of calcium from nonmitochondrial stores in the permeabilized cells, in agreement with results reported elsewhere (14). Ethanol (300 mM) caused a minor release of calcium into the medium but did not significantly affect either the rate or the extent of Ins(1,4,5)P₃-induced calcium release. The calcium release induced by ethanol stabilized at a slightly higher steady-state calcium level and probably represents some inhibition of the ATP-driven calcium pump in the endoplasmic reticulum (2). Both the change in this calcium set-point and the rate of calcium release after ethanol were insufficient to account for the calcium burst induced by ethanol in intact hepatocytes.

**Stimulation of Inositol Phosphate Production by Ethanol—** In further experiments we compared the effect of ethanol and vasopressin on the level of inositol phosphates in intact cells prelabeled with [³H]inositol for 90 min to label the inositol lipids. Most previous measurements of inositol phosphates have relied on a simple separation based on the number of phosphate groups on the inositol ring (41–43). However, it has recently been shown that biological tissues contain at

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1 J. B. Hoek, unpublished observations.
were compared to a vasopressin concentration which induces by an increase in the fluorescence signal.

For this reason, the effects of ethanol on InsP₃ levels were compared after 300 mM ethanol and after 1 or 40 nM vasopressin (Fig. 9). This is in keeping with the much greater potency of higher vasopressin concentrations to increase Ins(1,4,5)P₃ levels (42).

Fig. 10 shows time courses for the changes of inositol phosphates in hepatocytes treated with either 300 mM ethanol or 1 nM vasopressin. At these concentrations, ethanol and vasopressin act to increase the cellular levels of Ins(1,4,5)P₃ at a similar rate and to a similar extent (Fig. 10A). As a percentage of the control value before agonist addition, 300 mM ethanol increased Ins(1,4,5)P₃ by 79.8 ± 8.7% (n = 5) after 20 s, and with 1 nM vasopressin a maximal increase of 94.2 ± 19.3% (n = 4) was achieved within 1 min. In contrast to the similar rate of formation of Ins(1,4,5)P₃ with 300 mM ethanol or 1 nM vasopressin, the subsequent decline of this compound was very different for the two agonists. With vasopressin Ins(1,4,5)P₃ levels remained elevated throughout the period of the experiment, but with ethanol the level of Ins(1,4,5)P₃ had decayed back to the basal value by 2 min of ethanol treatment. This decay is also observed in the cytosolic free Ca²⁺ level (Fig. 9C). This is in keeping with the much greater potency of higher vasopressin concentrations to increase Ins(1,4,5)P₃ levels (42).
The transient nature of the \( \text{Ins}(1,4,5)P_3 \) increase with ethanol raises the question of its further metabolism. \( \text{Ins}(1,4,5)P_3 \) can be successively dephosphorylated through \( \text{InsP}_2 \), \( \text{InsP}_3 \), and finally to inositol (11, 13). It has recently been shown, however, that \( \text{Ins}(1,4,5)P_3 \) may be further phosphorylated to \( \text{Ins}(1,3,4,5)P_4 \), and it is possible that the other \( \text{InsP}_4 \) isomer, \( \text{Ins}(1,3,4)P_4 \), is derived from this \( \text{InsP}_3 \) (45). The HPLC elution profile contains a small peak which we tentatively identify as \( \text{InsP}_4 \) based on the similarity in ionic strength at which it is eluted compared to the results reported by others (30, 45); this peak contains only about 20% of the \( \text{InsP}_4 \) present at very much higher basal levels, did not change significantly during the first 2 min of stimulation even with 100 nM vasopressin, as noted previously (42).

The treatment of hepatocytes with ethanol is also accompanied by changes in the inositol phospholipid precursors of the inositol phosphates. Table I summarizes the results of our experiments with hepatocytes preincubated for 90 min with \( \text{InsP}_4 \) to label the monoester phosphate groups on the inositol ring of PtdIns(4)P and PtdIns(4,5)P_2. Ethanol (200 mM) caused a small but significant decline in PtdIns(4,5)P_2 within 60 s. At the same time, however, PtdIns(4)P increased markedly, presumably due to the simultaneous ethanol-dependent activation of PtdIns kinases that generate the polyphosphoinositides from PtdIns. There is also a significant increase in phosphatidic acid, presumably generated from diacylglycerol by diacylglycerol kinase. No significant changes in the levels of PtdIns and phosphatidylcholine occurred during the short time periods of this experiment.

**DISCUSSION**

Several lines of evidence indicate that the ethanol-induced increase in phosphorylase \( \alpha \) activity in hepatocytes is due to the mobilization of intracellular calcium, and not to other known effectors of phosphorylase kinase. In the first place, ethanol does not induce changes in cAMP level and has no effect on the dose-response curve of glucagon for phosphorylase activation. These data indicate that cAMP-dependent protein kinase is not activated by ethanol under these conditions. Second, a direct activation of phosphorylase kinase by ethanol, as reported by Singh and Wang (20) for the purified skeletal muscle enzyme, required higher concentrations than were used in the present study in intact hepatocytes. No significant effect of ethanol is observed in broken hepatocytes at concentrations up to 200 mM. In intact hepatocytes half-maximal activation of phosphorylase is obtained at 60–70 mM.
the cytosolic free Ca\(^{2+}\) level than the estimates reported here. Thus, in unloaded cells, the ethanol-induced changes in cytosolic Ca\(^{2+}\) are partly suppressed in the quin2-loaded cells. Further, ethanol and quin2 introduce a significant Ca\(^{2+}\) buffering capacity in the cytosol, and the ethanol-induced changes in cytosolic Ca\(^{2+}\) are partly suppressed in the quin2-loaded cells. Thus, in unloaded cells, ethanol can probably induce a significantly higher increase in the cytosolic free Ca\(^{2+}\) level than the estimates reported here indicate. A direct comparison of the ethanol-induced activation of phosphorylase in quin2-loaded and unloaded cells is difficult, because the loading procedure itself results in a significant increase in phosphorylase activity.

The effects of ethanol on phosphorylase in hepatocytes do not give maximal phosphorylase in quin2-loaded and unloaded cells is difficult, because the loading procedure itself results in a significant increase in phosphorylase activity.

The source of the calcium mobilized by ethanol is clearly intracellular, and the data strongly suggest that the calcium comes from the same pool as that mobilized by vasopressin and \(\alpha\)-adrenergic hormones in liver. This finding suggests the possibility that ethanol interacts with the signal transduction pathway used by these hormones to elevate cytosolic calcium. Our data do not indicate, however, that the effects of ethanol on intact cells can be attributed to a direct action on the membranes of the endoplasmic reticulum to activate Ca\(^{2+}\) efflux and/or inhibit the reuptake. In digitonin-permeabilized hepatocytes, the addition of ethanol caused only a minor and sluggish adjustment of the Ca\(^{2+}\) set-point (see Fig. 8), presumably as a consequence of the inhibition of the endoplasmic reticulum Ca\(^{2+}\) pump by ethanol.

Moreover, ethanol affected neither the rate nor the extent of Ins(1,4,5)P\(_3\) -induced calcium mobilization. These data indicate, therefore, that ethanol interacts with the signal transduction pathway at a point before the mobilization of calcium from the endoplasmic reticular stores.

The measurement of inositol phosphates and phosphoinositides (Fig. 10 and Table III) provided direct evidence that ethanol mobilizes intracellular calcium by activating the hormone-sensitive phosphoinositide-specific phospholipase C. Several arguments support the notion that the rapid accumulation of Ins(1,4,5)P\(_3\) after addition of ethanol is the direct cause of calcium mobilization. First, the change in Ins(1,4,5)P\(_3\) preceded the rise in calcium concentration. Second, the amount of Ins(1,4,5)P\(_3\) generated by ethanol correlated well with its potency as a calcium-releasing agent; this was demonstrated by the similar degree of Ins(1,4,5)P\(_3\) production induced by a low dose of vasopressin which was equivalent to 300 mM ethanol in its effects on calcium mobilization.

**Table III**

| Phospholipid | Change in \(^{32}\)P radioactivity in hepatocytes |
|-------------|-----------------------------------------------|
| PtdIns(4,5)P\(_2\) | 95 ± 2.0* |
| PtdIns(4)P | 114 ± 2.1* |
| PtdIns | 100 ± 1.0 |
| Phosphatidic acid | 112 ± 2.4* |

\(^*p < 0.05\) compared to control.

The estimated concentration increase for Ins(1,4,5)P\(_3\) in the ethanol-stimulated hepatocyte after 20 s was 0.20 ± 0.04 \(\mu\)M (mean ± S.D. of three individual experiments). In studies of permeabilized hepatocytes the half-maximal concentration for Ins(1,4,5)P\(_3\)-induced calcium release is in the range of 0.1 \(\mu\)M (14). Thus, both the kinetics of Ins(1,4,5)P\(_3\) formation and the amount generated after ethanol addition identify this compound as an appropriate physiologic mediator of calcium release. Third, the rise in Ins(1,4,5)P\(_3\) due to ethanol, unlike that induced by vasopressin, is transient, as is the calcium response. Thus, the level of Ins(1,4,5)P\(_3\) after ethanol stimulation closely parallels the cytosolic calcium at all phases of the transient.

Further support for a phospholipase C-mediated mechanism of ethanol action comes from the analysis of changes in substrate concentration, i.e. inositol lipids. A small but significant decrease in PtdIns(4,5)P\(_2\) occurred in response to ethanol addition, although the magnitude of the response varied and was often difficult to quantitate. This is not surprising in view of the low levels of Ins(1,4,5)P\(_3\) formed. Moreover, the steady state levels of the phospholipid intermediates in this pathway are affected by the activity of other enzymes, e.g. phosphomonoesterases, kinases, and phosphodiesterases. Receptor-mediated stimulation of phospholipase C is generally accompanied by an activation of the phosphoinositide kinases which regenerates the PtdIns(4,5)P\(_2\) from its precursor lipids (11-13). The elevation of the level of PtdIns(4)P in response to ethanol addition is an indication that the phosphoinositide kinases are also stimulated after ethanol addition.

Further evidence supporting an ethanol-induced activation of phospholipase C activity is the formation of phosphatidic acid, which can readily be generated from diacylglycerol in hepatocytes by diacylglycerol kinase.

Thus, the data presented here provide strong evidence that ethanol initiates the activation of the same pathway employed by vasopressin and other calcium-mediated hormones in hepatocytes and that it generates the same second messenger signals.

A major difference between the ethanol-induced and the hormone-receptor-mediated responses is the transient nature of the former signal. The ethanol-induced calcium increase decayed to basal levels over a 2-4 min period, despite the continued presence of ethanol and even when its oxidation through alcohol dehydrogenase was inhibited. The decay of the calcium signal is preceded by a decline of both isomers of InsP\(_3\) to basal levels (Fig. 10) and hence represents a...
deactivation of phospholipase C. The calcium mobilized by Ins(1,4,5)P3 is reaccumulated in the same stores, where it is available for a renewed challenge, either by a subsequent addition of ethanol or by a hormonal stimulus (see Figs. 3 and 7). This situation is markedly different from that induced by hormones, where a partially elevated level of Ins(1,4,5)P3 persists and the calcium stores are not replenished until the hormone is removed or an antagonist is added.

Further studies will be required to identify possible mechanisms of the activation of phosphoinositide-specific phospholipase C by ethanol. The hormonal activation of this enzyme involves the transmembrane interaction with a hormone receptor, possibly mediated by a GTP-binding coupling protein similar to, but not identical with, the Gi and Gq receptors-adenylate cyclase coupling (48). It is conceivable, however, that acti-

REFERENCES

1. Tarasci, T. F., and Rubin, B. (1985) Biochim. Biophys. Acta 839, 243–245
2. Murphy, E., Coll, K., Rich, T. L., and Williamson, J. R. (1980) J. Biol. Chem. 255, 6690–6696
3. Joseph, S. K., and Williamson, J. R. (1983) J. Biol. Chem. 258, 10425–10432
4. Charest, R., Blackmore, P. F., Berthon, B., and Exton, J. H. (1983) J. Biol. Chem. 258, 8769–8775
5. Reinhart, P. H., Taylor, W. M., and Bygrave, F. L. (1983) Biochem. J. 214, 405-412
6. Williamson, J. R., Cooper, R. H., Joseph, S. K., and Thomas, A. P. (1985) Am. J. Physiol. 248, C203-C216
7. Nakamura, Y. (1984) Nature 309, 693–698
8. Berridge, M. J. (1984) Biochem. J. 220, 345–360
9. Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F., and Williamson, J. R. (1984) J. Biol. Chem. 259, 3077–3081
10. Kishimoto, A., Takei, Y., Mori, T., Kikkawa, U., and Nakamura, Y. (1980) J. Biol. Chem. 255, 2273–2276
11. Grimes, R. J., Johnson, D. E., and Campanile, C. P. (1984) J. Biol. Chem. 259, 3283-3292
12. Carlson, G. M., Bechtle, P. J., and Graves, D. J. (1979) Adv. Enzymol. 45, 29–109
13. Walsh, D. A., and Krebs, E. G. (1973) in The Enzymes (Boyer, P. D., ed.) Vol. 6, pp. 555–561, Academic Press, Orlando, FL
14. Cohen, P. (1975) Eur. J. Biochem. 54, 1–14
15. Singh, T. J., and Wang, J. H. (1979) J. Biol. Chem. 254, 8466–8472
16. Vranick, S., Weidlich, W., DeWulf, H., and Merlevede, W. (1979) J. Biol. Chem. 254, 101, 51–68
17. Doornweerd, D. D., Tan, A. W. H., and Nutall, F. Q. (1982) Mol. Cell. Biochem. 47, 45–53
18. Berry, M. N., and Friend, D. S. (1969) J. Cell Biol. 43, 506–520
19. Meijer, A. J., Gimpel, J. A., de Leeuw, G. A., Tager, J. M., and Williamson, S. J. (1975) J. Biol. Chem. 250, 778–783
20. Tsien, R. Y. (1981) Nature 290, 527–538
21. Tsien, R. Y., Pozzan, T., and Rink, T. J. (1982) J. Cell Biol. 94, 325–334
22. Arvan, I., DiVirgilio, F., Beltran, M., Tsien, R. Y., and Pozzan, T. (1985) J. Biol. Chem. 260, 2719–2727
23. John, F., Wirtz, K. W. A., Schotman, P., and Gispen, W. H. (1979) FEMS Lett. 105, 110–114
24. Irvine, R. F., Angard, E. E., Letcher, A. J., and Downes, C. P. (1982) J. Biol. Chem. 257, 505–510
25. Batty, I. R., Nahorski, S. R., and Irvine, R. F. (1985) Biochem. J. 232, 211–216
26. Gilman, A. G., and insure, F. Q. (1972) Anal. Biochem. 47, 20–27
27. Ulrich, R. J., Janosi, A. M., and Graves, D. J. (1979) J. Biol. Chem. 254, 3166–3169
28. Gilman, A. G., and Murrell, F. (1974) Methods Enzymol. 38, 49–61
29. Blackmore, P. F., Hughes, H. P., Charest, R., Shuman, E. A., and Ivan, J. R. (1983) J. Biol. Chem. 258, 10486–10494
30. Hoek, J. B. (1986) Ann. N. Y. Acad. Sci., in press
31. Williamson, J. R., and Tischer, M. (1979) in Biochemistry and Pharmacology of Ethanol (Macjewicz, E., and Noble, E., ed) Vol. 1, pp. 167–189, Plenum Publishing Corp., New York
32. Rogozin, R., and Griffin, C. (1980) in Biochemistry and Pharmacology of Ethanol (Macjewicz, E., and Noble, E., ed) Vol. 1, pp. 65–85, Plenum Publishing Corp., New York
33. Dalziel, K., and Dickinson, F. M. (1966) Biochem. J. 100, 34–46
34. Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F., and Putney, J. W. (1981) Nature 290, 63–68
35. Joseph, S. K., Coll, K. E., Thomas, A. P., Rubin, R., and Williamson, J. R. (1985) J. Biol. Chem. 260, 12506–12515
36. Berridge, M. J. (1983) Biochem. J. 213, 573–586
37. Thomas, A. P., Alexander, J., and Williamson, J. R. (1984) J. Biol. Chem. 259, 5674–5684
38. Crossnet, R., Pric, V., Exton, J. H., and Blackmore, P. F. (1985) Biochem. J. 227, 79–90
39. Irvine, R. F., Letcher, A., Lander, D. J., and Downes, C. P. (1984) Biochem. J. 223, 227–243
40. Irvine, R. F., Letcher, A., and Helsop, J. P., and Berridge, M. J. (1986) Nature 320, 631–634
41. Burgess, G. M., McKinney, J. S., Irvine, R. F., and Putney, J. W. (1986) Biochem. J. 232, 237–248
42. Thomas, A. P., Hoek, J. H., and Rubin, R. (1986) Ann. N. Y. Acad. Sci., in press
43. Ulrich, R. J., Pric, V., Jiang, H., and Exton, J. H. (1986) J. Biol. Chem. 261, 2140–2146
44. Rubin, R. A., and Molinoff, P. B. (1983) J. Pharmacol. Exp. Ther. 227, 551-556
45. Kiss, Z., and Thachuk, V. A. (1984) Eur. J. Biochem. 142, 323–329
46. Sato, T., Lee, J. M., and Tabakoff, B. (1985) J. Neurochem. 44, 1037–1044
47. Cooper, R. H., Coll, K. E., and Williamson, J. R. (1985) J. Biol. Chem. 260, 2891–2896
48. Lynch, C. J., Charest, R., Bocckino, S., Exton, J. H., and Blackmore, P. F. (1985) J. Biol. Chem. 260, 2845–2851

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REFERENCE

1. Tarasci, T. F., and Rubin, E. (1985) Lab. Invest. 52, 120–131
2. Honnapra, B. C., Waring, A. J., Exton, J. B., Rottenberg, H., and Rubin, E. (1985) J. Biol. Chem. 257, 10141–10146