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ETHANOL-INDUCED STIMULATION OF PHOSPHOINOSITIDE TURNOVER AND CALCIUM INFLUX IN ISOLATED HEPATOCYTES*

RAPHAEL RUBIN and JAN B. HOEK†

Department of Pathology and Cell Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, U.S.A.

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Abstract—Ethanol has been shown to mobilize intracellular calcium in isolated rat hepatocytes by activation of phosphoinositide-specific phospholipase C. However, addition of ethanol to ^{32}P-labeled hepatocytes resulted in a rapid increase in the level of [^{32}P]phosphatidylinositol 4-phosphate over a period of 2 min, concomitant with a small decrease in [^{32}P]phosphatidylinositol 4,5-bisphosphate and an increase in [^{32}P]phosphatidic acid levels. These results indicate that polyphosphatidylinositol metabolism was stimulated by ethanol simultaneously with the activation of phospholipase C. Ethanol also caused a transient increase in the influx of extracellular calcium into quin 2-loaded hepatocytes over a similar period of time. The results demonstrate that ethanol, in common with calcium-mobilizing hormones, directly or indirectly stimulated polyphosphoinositide regeneration and allowed for increased movement of calcium across the hepatocyte plasma membrane.

In a recent study [1] we reported effects of ethanol and other hydrophobic solvents on calcium homeostasis in isolated hepatocytes. The addition of ethanol to intact hepatocytes was found to induce a mobilization of calcium from an intracellular, hormone-sensitive storage site, thereby resulting in a transient increase in cytosolic calcium levels. The release of calcium was associated with an increased level of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)‡ in the cell, a compound known to act as a second messenger in receptor-mediated release of calcium from stores presumably located in the endoplasmic reticulum (see Refs. 2 and 3 for reviews). Furthermore, ethanol caused only a minor release of calcium from permeabilized hepatocytes and did not affect the $Ins(1,4,5)P_3$-releasable calcium pools directly. The results of these studies indicated that ethanol can alter cellular calcium homeostasis by activating the hormone-sensitive, phosphoinositide-specific phospholipase C located in the plasma membrane of hepatocytes.

The interaction of calcium-mobilizing hormones (e.g. vasopressin, α_1-adrenergic agonists) with hepatocytes not only causes the activation of phospholipase C, but concomitantly stimulates other processes that help ensure a coordinated second messenger response of the cells [4]. One of these processes is the regeneration of $PtdIns(4,5)P_2$ by activation of the PtdIns kinases which phosphorylate the 4′- and 5′-positions on the inositol ring [3, 5]. The mechanism of activation of these kinases is not clear. It could be a direct result of the hormone–receptor interaction or a secondary consequence of the depletion of $PtdIns(4,5)P_2$ levels, i.e. activation of the enzyme by product depletion. Alternatively, it could be mediated by one or more of the second messenger signals which are generated by $PtdIns(4,5)P_2$ degradation.

In addition to activating phosphoinositide-specific kinases, hormonal stimulation also induces the opening of a receptor-linked calcium channel in the plasma membrane of hepatocytes [6, 7]. This channel allows an increased rate of calcium influx which helps maintain an elevated level of cytosolic free calcium after intracellular stores are depleted. It has been suggested that receptor-operated calcium channels are activated by $Ins(1,3,4,5)P_4$ [8, 9]. Alternatively, receptor-operated ion channels may be regulated through distinct G-proteins; however, evidence for either of these mechanisms is not available in hepatocytes.

Ethanol presumably activates phospholipase C by a mechanism that bypasses the specific hormone receptors [1]. It was of interest, therefore, to assess the ability of ethanol to stimulate these other activities associated with hormone–receptor interactions. In this paper, we report that ethanol, in concentrations that induce activation of the phosphoinositide-specific phospholipase C, also activated the regeneration of polyphosphoinositides as well as the

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† To whom correspondence should be sent: Jan B. Hoek, Ph.D., Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107.

‡ Abbreviations: $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $InsP_2$, inositol 1,4-bisphosphate; InsP, inositol phosphate; $Ins(1,3,4,5)P_4$, inositol 1,3,4,5-tetrakisphosphate; $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; DG, diacylgylcerol; and EGTA, ethyleneglycolbis (aminoethylether)tetra-acetate.

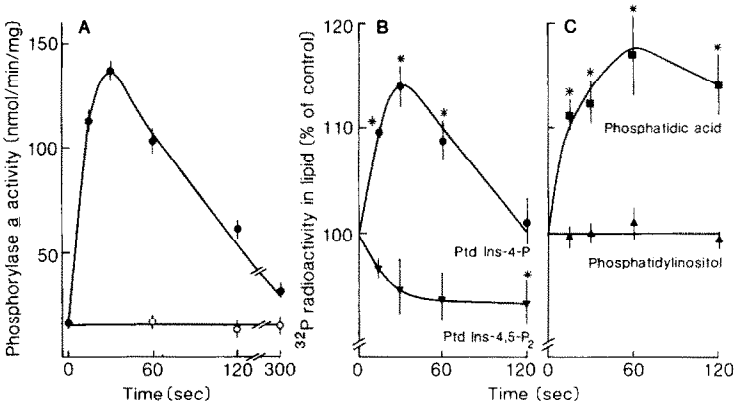

Fig. 1. Effects of ethanol on phosphorylase activity and inositol phospholipid levels in isolated hepatocytes. Hepatocytes were incubated for 90 min without (A) or with (B and C) $^{32}P_i$ (10 μCi/ml) before the addition of 200 mM ethanol. At the indicated times, samples were taken for phosphorylase or phospholipid determination as described under Materials and Methods. Each point is the mean ± SEM of values obtained from four to five separate experiments with individual measurements corrected for PC content and expressed as the percentage change of the basal level within that experiment. Key: (A) phosphorylase *a* activity: control (○), ethanol (●); (B) [^{32}P]-radioactivity of PtdIns(4,5)P_2 and PtdIns(4)P; (C) [^{32}P]radioactivity of PA and PtdIns. *$P < 0.05$ compared to time zero.

calcium influx across the plasma membrane. A preliminary account of some of these data has appeared elsewhere [10].

MATERIALS AND METHODS

Carrier-free [^{32}P]ATP and [^{32}P]orthophosphoric acid were purchased from the Amersham Corp., Arlington Heights, IL. Other chemicals and biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO, and from the Fisher Scientific Co., Pittsburgh, PA, and were of the highest purity commercially available.

Male Sprague–Dawley rats fed *ad lib.* were used for the preparation of isolated hepatocytes by collagenase perfusion as described by Meyer *et al.* [11]. Cell incubations were carried out in a shaking water bath at 37° in stoppered 25-ml flasks in Krebs–Ringer bicarbonate buffer, pH 7.4, supplemented with 2% dialyzed bovine serum albumin and 15 mM glucose. The gas phase contained 95% O_2/5% CO_2. For isotopic labeling, the cells were preincubated for 90 min in the same medium containing 10 μCi/ml $^{32}P_i$ (sp. act. of P_i was 8.3 μCi/μmol) and 5 mg cell protein/ml. For the measurement of ^{32}P-labeled phospholipids, 0.15 ml of cell suspension was quenched with 0.56 ml chloroform–methanol–HCl (200:100:5). Quin 2-loaded cells were prepared by the method of Tsien [12] and incubated in the cuvet of a Perkin–Elmer MPF-44B spectrofluorometer using a wavelength pair of 339 nm and 492 nm as described earlier [1]. Phosphorylase *a* activity was determined essentially as described by Gilboe *et al.* [13].

^{32}P-Labeled lipids were separated by thin-layer silica-gel chromatography, and the radioactivity in each lipid was quantitated by liquid scintillation counting, as described previously [1]. The final radioactive content in the lipids varied in different experiments and ranged from 150 to 400 cpm for PtdIns(4,5)P_2 and PtdIns(4)P and from 500 to 1000 cpm for PA. The amounts of polyphosphoinositides were calculated from the specific activity of the $^{32}P_i$ added, assuming complete isotopic equilibrium of PA and of the 4- and 5-phosphate groups on the inositol ring. Protein concentrations were determined by the biuret method. Statistical significance was calculated by Student's *t*-test.

RESULTS

Turnover of polyphosphoinositides. Figure 1 illustrates the effect of ethanol on the level of inositol phospholipids and phosphatidic acid (PA) in isolated hepatocytes. The cells were labeled by preincubation with $^{32}P_i$ for 90 min, a period sufficient to obtain equilibrium labeling of the monoester phosphate groups attached to the inositol ring of PtdIns(4)P and PtdIns(4,5)P_2 [5]. Subsequent short-term changes in the ^{32}P content of the polyphosphoinositides and PA can be presumed to represent changes in the total amount of these substances. The experiment of Fig. 1A shows that these preincubation conditions did not impair the capacity of the cells to respond to ethanol. Addition of 200 mM ethanol rapidly activated phosphorylase *a*. The activity was maximal by 30 sec and declined gradually over the ensuing 5 min. We have shown earlier that activation of phosphorylase *a* occurs over an ethanol concentration range of 20–300 mM [1]. Ethanol causes a rapid accumulation of Ins(1,4,5)P_3 which peaks within 20–30 sec and then declines to baseline over a period of 2 min [1]. Figure 1B demonstrates that there was a small, but significant, decrease in the level of PtdIns(4,5)P_2 over the same time period which stabilized at 94 ± 2% of the control incubations. PA levels increased substantially, to reach a value of 117 ± 3.3% of control values after 60 sec (Fig. 1C). PA is presumed to be formed, in part, from diacylglycerol which is generated by the degradation of the phosphoinositides [2, 5].

Table 1. Stoichiometry of phospholipid interconversions after stimulation of isolated hepatocytes with ethanol

Reaction time (sec)	ΔPtdIns(4)P	ΔPtdIns(4,5)P_2	ΔPA	Σ
	(pmol/mg protein)			
15	4.0 ± 0.1	−1.4 ± 0.3	11.1 ± 1.4	13.7
30	5.8 ± 0.7	−2.5 ± 0.5 −3± 0.9	12.3 ± 2.1	15.6
60	3.6 ± 0.7		17.3 ± 3.3 14. 2.6	17.9
120	0.4 ± 0.8	−3.1 ± 0.9		11.5

Data were obtained from the experiments shown in Fig. 1. Amounts of phospholipids were calculated from the changes in [^{32}P]radioactivity, assuming equilibrium labeling of the phosphate groups in PA and in the 4- and 5-positions of the inositol ring of PtdIns(4)P and PtdIns(4,5)P_2. Total cellular contents of phospholipids at zero time were: PtdIns(4)P, 43.5 ± 6.5 pmol/mg protein; PtdIns(4,5)P_2, 24.6 ± 3.7 pmol/mg protein; and PA, 131 ± 5.4 pmol/mg protein (mean ± SEM for five separate experiments). Σ represents the algebraic sum of changes in PtdIns(4)P, PtdIns(4,5)P_2 and PA.

These findings are consistent with the activation of phospholipase C by ethanol [1]. The stimulation of phospholipase C activity was accompanied, however, by an increase rather than a decrease in the level of PtdIns(4)P; the level of this compound rose transiently to a peak of 114 ± 2% of control at 30 sec after ethanol addition and declined to control levels by 2 min (Fig. 1B). No significant changes could be detected in the level of PtdIns or PC over the 2-min period after ethanol addition. These observations can be compared with previous studies of vasopressin-induced lipid breakdown in hepatocytes over short time periods [5]; however, for vasopressin concentrations that are equipotent with ethanol (see Ref. 1), PtdIns(4,5)P_2 degradation was more substantial and PtdIns(4)P levels generally were found to decrease as well. These differences indicate that the stimulation of PtdIns kinase relative to phospholipase C is greater after ethanol treatment than after stimulation with vasopressin.

Further insight into the ethanol-induced phosphoinositide interconversions can be gained from a consideration of the stoichiometries of the metabolic changes shown in Table 1. Estimates of the amounts of phosphoinositides present in the cell were made assuming complete equilibration of the 4- and 5-phosphate groups with $^{32}P_i$ and neglecting the small amount of ^{32}P introduced through PI and the isotopic dilution in cellular ATP. PtdIns(4)P may be formed either from PtdIns by the action of PtdIns kinase or by the action of a 5′-phosphomonoesterase hydrolyzing PtdIns(4,5)P_2. The decline in the level of PtdIns(4,5)P_2 (2.5 ± 0.5 pmol/mg protein) 30 sec after ethanol addition, however, was insufficient to account for the increase in PtdIns(4)P (5.8 ± 0.7 pmol/mg after 30 sec). Moreover, although other sources of PA cannot be excluded, it is likely that the polyphosphoinositides are also the precursors of PA which was formed to the extent of 12.3 ± 2.1 pmol/mg after 30 sec of ethanol treatment [14]. The net flux through this pathway, therefore, appears to be from PtdIns to PtdIns(4)P and PtdIns(4,5)P_2 and through diacylglycerol to phosphatidic acid. It is also possible that some of the PA derives from diacylglycerol generated by direct phospholipase C-mediated hydrolysis of PtdIns(4)P [14]. In either case, the potential synthesis of PtdIns(4)P from PtdIns would amount to at least 15.6 pmol/mg in 30 sec, and could be considerably higher if significant amounts of diacylglycerol accumulate or are converted into other products. An equivalent amount of inositol phosphates must also have been formed by the degradation of PtdIns(4,5)P_2. However, the inositol phosphates that accumulated over this 30-sec period were only a small fraction of the total amount of polyphosphoinositides which were apparently degraded by phospholipase C. In a series of three experiments with hepatocytes prelabeled with [^{3}H]*myo*-inositol as described earlier [1], addition of 300 mM ethanol caused an increase in the level of inositol phosphates as follows: Ins(1,4,5)P_3, 0.43 ± 0.13 pmol/mg; Ins(1,3,4)P_3, 0.63 ± 0.12 pmol/mg; InsP_2, 3.16 ± 0.95 pmol/mg (A. P. Thomas and J. B. Hoek, unpublished observations). It is likely that a large fraction of inositol phosphates is further degraded to InsP and free *myo*-inositol, which would not have been detected under our assay conditions.

The concentration dependency of ethanol-induced activation of the PtdIns kinases is shown in Fig. 2. Increases in the levels of PtdIns(4)P and phosphatidic acid were observed at all concentrations studied. The level of PtdIns(4,5)P_2, while decreasing upon addition of 200 mM ethanol, actually increased at higher levels of ethanol. The increase in PA production in Fig. 2 indicates that phospholipase C was further stimulated with higher ethanol levels. The conversion of PtdIns(4)P to PtdIns(4,5)P_2 also appeared to be stimulated even more at high ethanol concentration, resulting in an increased level of the latter.

The data of Figs. 1 and 2 suggest the possibility that ethanol has an effect on both phospholipase C and the enzymes catalyzing the interconversion of PtdIns to PtdIns(4)P and PtdIns(4,5)P_2. The accumulation of the polyphosphoinositides could be stimulated either by activation of PtdIns kinase and PtdIns(4)P kinase, or by inhibiting the activity of

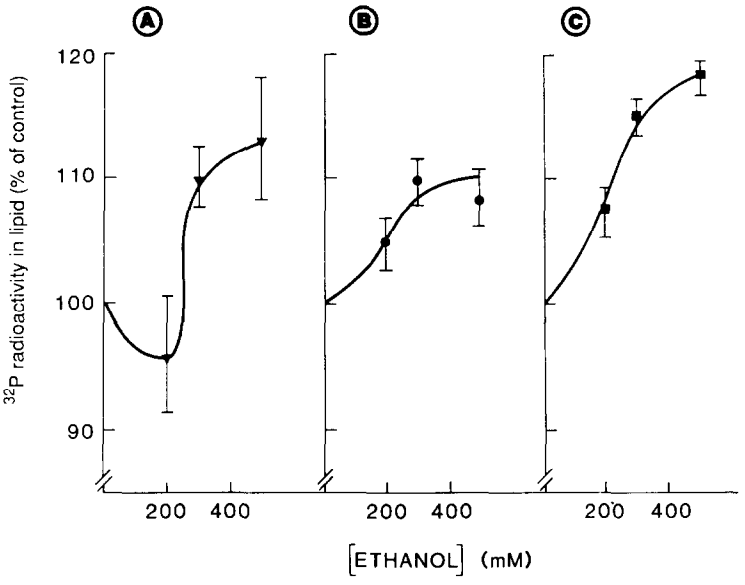

Fig. 2. Effect of ethanol concentration on the accumulation of PtdIns(4)P and phosphatidic acid in isolated hepatocytes. After a 90-min preincubation with $^{32}P_i$, isolated hepatocytes were treated for 30 sec with the indicated concentration of ethanol. Data are the mean ± SEM of values obtained from three separate experiments and expressed as the percentage change of basal levels, within each experiment, of [^{32}P]radioactivity in PtdIns(4,5)P_2 (A), PtdIns(4)P (B), and PA (C).

phosphomonoesterases acting on PtdIns(4,5)P_2 and PtdIns(4)P. Our data do not distinguish between these possibilities.

The activity of PtdIns kinase was tested directly in hepatocytes permeabilized by pretreatment with digitonin, as described in Ref. 15. Under these conditions, the rate of incorporation of phosphate from [^{32}P]ATP into polyphosphoinositides was not affected by ethanol in concentrations up to 300 mM (data not shown). Phosphomonoesterase activity is more difficult to assess in permeabilized hepatocytes. However, in cell preparations prelabeled with [^{3}H]inositol prior to permeabilization, degradation of PtdIns(4,5)P_2 approximately matched the formation of inositol phosphates, indicating that, under the conditions used, phosphomonoesterase did not contribute to a large extent to the degradation of polyphosphoinositides.

Calcium influx across the plasma membrane. A second plasma membrane-associated process that is known to respond to hormonal stimulation is calcium influx [6, 7]. In our earlier studies [1], we reported that the transient mobilization of calcium by ethanol did not require the presence of extracellular calcium, as both the increase in cytosolic free calcium and the subsequent recovery were unaffected by the presence of EGTA in the medium. Further studies shown in Fig. 3 show that a subsequent addition of more ethanol or a calcium-mobilizing hormone can induce a renewed calcium burst in normal calcium-containing medium (Fig. 3a). However, when cells were pretreated with EGTA, although the initial Ca^{2+} transient induced by ethanol was unaffected, a subsequent mobilization of calcium by ethanol was reduced markedly (Fig. 3b). In control incubations, EGTA by itself could not deplete the hormone-sensitive calcium stores over this time of exposure (data not shown). These data suggest that the ethanol treatment partly depletes the Ins(1,4,5)P_3 sensitive calcium stores unless these are replenished by the influx of extracellular calcium. The question is raised, therefore, whether ethanol opens up a calcium channel in the plasma membrane to facilitate the influx of calcium, similar to the receptor-mediated stimulation of calcium influx.

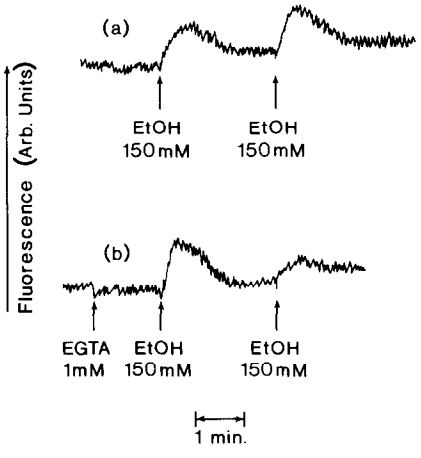

Fig. 3. Contribution of extracellular calcium to ethanol-induced calcium mobilization. Hepatocytes were loaded with quin 2 as described in Ref. 1. A 150 mM concentration of ethanol was added, where indicated, in the presence (a) or absence (b) of extracellular calcium. The recordings were obtained from one of several similar experiments.

Calcium influx was measured in quin 2-loaded cells, essentially following the method of Joseph *et al.* [7]. In this procedure, calcium (1 mM) is added to the medium after preincubating the cells in a calcium-free medium and the rate of Ca^{2+} influx is determined from the initial rate of the fluorescence increase due to intracellular quin 2-Ca^{2+}. At the intracellular concentration of quin 2 employed in these experiments (0.5 to 1.0 mM), the larger part of calcium entering the cytosolic cell compartment was bound to quin 2 and only a minor fraction contributed to the increase in cytosolic free calcium levels.

The increase in cytosolic Ca^{2+} measured with this procedure gives a minimum estimate of the rate of Ca^{2+} influx across the plasma membrane. The incoming calcium is partly disposed of, either by accumulation into intracellular storage sites or by release from the cell, e.g. through the plasma membrane calcium pump. The rate of disposition of the cytosolic Ca^{2+} could be affected by the changes in intracellular calcium handling induced by ethanol or vasopressin. In the procedure of Joseph *et al.* [7], these interferences are minimized in part by the buffering action of quin 2 in the cytosol and in part by assessing the initial rate of the fluorescence change, which is linear for at least 10–15 sec.

In the experiments of Table 2, $CaCl_2$ (1 mM) was added to the cells at different times after exposure to ethanol (300 mM) or vasopressin (1 nM). We have shown earlier [1] that this concentration of vasopressin is sufficient to give an increase of Ins(1,4,5)P_3 equivalent to that obtained with ethanol; the effects of the two agents differed, however, in that the ethanol-induced Ins(1,4,5)P_3 accumulation declined to basal levels over 2 min, whereas that induced by vasopressin was maintained. Table 2 illustrates the effects of these agents on the rate of calcium influx. The addition of calcium to untreated hepatocytes induced an influx of calcium at a rate of approximately 60 pmol/min/mg protein as detected by the increase in quin 2 fluorescence. After vasopressin treatment, the rate of calcium influx doubled over the first minute and remained elevated for a period of at least 5 min. Ethanol treatment also induced a significant increase in calcium influx rates, to approximately twice the control rate. The effect was not persistent, however, and had declined back to basal levels by 5 min.

DISCUSSION

The data presented here demonstrate that ethanol activated not only the hormone-sensitive phospholipase C, as reported earlier [1], but also some of the secondary activities that are normally associated with the hormonal stimulation of hepatocytes. Both the resynthesis of the polyphosphoinositides and the stimulation of plasma membrane calcium influx are activities required for the maintenance of the activated state of the cell. As such, a close interrelationship with the primary event of phospholipase C activation is to be expected. However, the mechanisms by which the activation of PtdIns kinases and calcium influx are integrated with the hormone-stimulated phospholipase C activation in hepatocytes have not been identified. It is difficult, therefore, to identify the mechanisms by which ethanol could activate the PtdIns phosphorylation and the calcium influx across the plasma membrane. Since both activities are membrane-bound, ethanol may exert its effect by a non-specific action on membrane-bound enzymes mediated through a disordering effect on the phospholipid bilayer structure. If this were true, however, it is difficult to understand why the effects were transient. The disordering of the membrane structure persists as long as ethanol is present; by contrast, both the calcium influx rate and PtdIns phosphorylation returned to baseline after 2 min, despite the continued presence of ethanol in the system (ethanol metabolism is too slow to

Table 2. Activation of Ca^{2+} influx by ethanol and vasopressin in isolated hepatocytes

Agonist	Reaction time (min)	Ca^{2+} influx rate (pmol/min/mg protein)	P
None		61 ± 7.2	
Ethanol	1	131 ± 1.7	<0.01
(300 mM)	2	94 ± 10.7	<0.02
	5	63 ± 3.3	NS*
Vasopressin	1	122 ± 29.8	<0.02
(1 nM)	2	93 ± 12.9	<0.02
	5	123 ± 14.5	<0.01

Isolated hepatocytes were loaded with quin 2 and incubated in a calcium-free medium. After temperature equilibration, agonist was added, followed after 1, 2 or 5 min by $CaCl_2$ (1 mM). The rate of increase in quin 2 fluorescence upon Ca^{2+} addition was followed at a wavelength pair of 339 nm and 492 nm. Calibration of the quin 2 signal was carried out in parallel incubations with unloaded cells and known amounts of quin 2. Data are mean ± SEM for three independent experiments. Statistical significance was calculated by Student's *t*-test, compared with the rate with no agonist added.

* NS, not significant at 95% confidence level.

account for the transient nature of the stimulation; see Ref. 1).

The activation of calcium influx and PtdIns phosphorylation may be a secondary consequence of the stimulation by ethanol of phospholipase C, mediated through one of the metabolites formed. Some of these metabolites can be excluded on kinetic grounds; for instance, the accumulation of $InsP_2$ and InsP showed a distinct lag time which was not reflected in the formation of PtdIns(4)P, and phosphatidic acid levels remained elevated even when the calcium influx rate had decayed to baseline. A current candidate for the control of receptor-operated calcium channel activity is $Ins(1,3,4,5)P_4$ [8, 9]. However, this compound has not been shown to alter plasma membrane calcium fluxes in hepatocytes.

A third model for the ethanol-induced activation of calcium influx and PtdIns phosphorylation might be suggested if these activities were directly coupled to hormone–receptor occupancy through a G-protein. There are indications that some G-protein-mediated processes may be specific targets of ethanol action. Ethanol is known to affect adenylate cyclase activity in different types of cells, at least in part by an effect at the level of the G-protein–adenylate cyclase interaction [16]. Similarly, we have argued elsewhere [17] that the control of phospholipase C by putative G-proteins is a potential target of ethanol's effects on hepatocytes. A disturbance by ethanol of the protein–protein interactions within the membrane may be the basis of all these activating effects. Detailed studies remain to be carried out for the activation of phospholipase C and for the processes reported here to be activated by ethanol. It also remains to be explained why the effect of ethanol is so transient under conditions where the hormonally induced activation of these processes persists. Further studies to clarify these aspects await the preparation of an isolated plasma membrane fraction in which the effects of ethanol on the hormone-sensitive phospholipase C are maintained.

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