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EFFECT OF ETHANOL ON AMYLASE SECRETION AND CELLULAR CALCIUM HOMEOSTASIS IN PANCREATIC ACINI FROM NORMAL AND ETHANOL-FED RATS

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Abstract—The effects of ethanol on stimulus–secretion coupling were assessed by studying amylase release, Ca^{2+}-homeostasis, and changes in physical properties of membranes in isolated rat pancreatic acini. In acini from normal rats, ethanol (50 mM and above) *in vitro* caused a dose-dependent stimulation of amylase release and an increase in cytosolic free Ca^{2+} concentration. Ethanol did not affect amylase secretion stimulated by cholecystokinin-octapeptide (CCK_8), a secretagogue that acts by increasing cytosolic free Ca^{2+} levels, but did potentiate the secretion of amylase induced by vasoactive intestinal peptide (VIP) which raises intracellular cAMP. Ethanol also increased the rate of $^{45}Ca^{2+}$ exchange. In acini labeled with the spin-probe 12-doxyl stearic acid, ethanol disordered the pancreatic plasma membranes. By contrast, in acini from animals that had chronically (6–7 weeks) ingested ethanol, the membranes were resistant to this disordering effect of ethanol. Chronic ethanol feeding lowered the total cellular calcium content and ionophore (A23187)-releasable pools of acinar calcium (11 and 24% respectively), and led to a 15–30% decrease in the rate of $^{45}Ca^{2+}$ exchange. Chronic ethanol ingestion also lowered the basal rate of amylase secretion, but ethanol *in vitro* stimulated amylase secretion more than in control preparations. However, these differences in basal and ethanol-induced amylase secretion were not accompanied by corresponding changes in intracellular free Ca^{2+}. The data suggest that ethanol perturbs cell membranes and also disturbs cellular Ca^{2+} homeostasis. These effects may explain its actions as a weak Ca^{2+}-mediated secretagogue. However, the membrane alterations induced by chronic ethanol feeding do not prevent the ethanol-induced interference with cellular calcium homeostasis.

Chronic ethanol ingestion is associated with the development of pancreatitis in man [1]. Yet, the mechanisms by which ethanol exerts effects on the exocrine pancreas have not been established, and few systematic biochemical studies of the effects of ethanol on cellular processes involved in stimulus–secretion coupling have been reported. Long-term ethanol ingestion in rats [2–4] and in humans [5] has been reported to cause morphologic changes similar to those in the liver [6]. In a study of the effect of short-chain aliphatic alcohols on pancreatic acinar cells from the guinea pig [7], ethanol concentrations up to 0.4 M had no effect on basal or vasoactive intestinal peptide (VIP)-stimulated amylase secretion. Higher levels of ethanol slightly stimulated basal secretion, but inhibited amylase secretion stimulated by secretagogues which mobilize cellular calcium, such as cholecystokinin-octapeptide (CCK_8) and carbamylcholine [7, 8]. In addition, ethanol potentiated the activation of adenylate cyclase by VIP [7].

In recent studies on isolated liver cells [9], we reported that ethanol can cause a transient mobilization of intracellular calcium, resulting in the activation of calcium–calmodulin-dependent enzymes in the cytosol. These effects of ethanol were mimicked by other aliphatic alcohols, general anesthetics, and several other hydrophobic compounds in high concentrations, and were presumably caused by the interaction of these compounds with cellular membranes. These studies suggest the possibility that effects of ethanol on stimulus–secretion coupling in pancreatic acini might also be mediated by changes in cellular calcium homeostasis.

The exocrine pancreas has been demonstrated to have relatively low activities of ethanol-oxidizing enzymes [1, 10]. Therefore, the effects of ethanol on pancreatic acini are not likely to be due to its metabolism in the pancreas. Earlier studies from our laboratory [11–13] have emphasized the effects of acute and chronic ethanol treatment on structural features of biological membranes. Chronic ethanol ingestion induces an adaptive change in membranes that makes them resistant to the disordering effect of ethanol [10–14]. It might be predicted, therefore, that an interference of ethanol *in vitro* with stimulus–secretion coupling might be altered in preparations from rats that have been fed an ethanol-containing diet.

In the studies reported here, we have investigated these hypotheses by studying the effects of chronic ethanol administration *in vivo* and of ethanol *in vitro* on amylase secretion, calcium homeostasis and membrane order parameters in isolated rat pancreatic acini.

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MATERIALS AND METHODS

Animals: Chronic ethanol feeding. Male Sprague–

Dawley rats (Charles River Breeding Laboratories), initially weighing 110–130 g, were fed a liquid diet (Bioserv Inc., Frenchtown, NJ) in which ethanol provided 36% of the total calories [15]. Littermate controls were pair fed the same diet, except that carbohydrates isocalorically replaced ethanol. Rats were maintained on this diet for 6–7 weeks, ethanol consumption averaging 14–16 g/kg body wt/day. Before the rats were killed (9:30–10:30 a.m.), they were fasted overnight, during which time the ethanol-fed rats were given water containing ethanol (5 g/l), whereas pair-fed controls received only water. All other rats were chow-fed, Sprague–Dawley males (300–350 g) fasted overnight, water being provided.

Preparation of isolated pancreatic acini. The procedure for the preparation of isolated pancreatic acini was according to Williams *et al.* [16], with the following modifications. The starting material was 0.6 to 1.5 g pancreas obtained from one or two rats. The medium used for the isolation was a Krebs–Hensleit bicarbonate medium, modified by adding soybean trypsin inhibitor (0.1 mg/ml) and minimal Eagle's medium amino acid supplement (Gibco) [16]. The dissociation medium contained 60–75 units/ml of collagenase (CLSPA-5275 Worthington Biochemicals) and 1.0 mg/ml hyaluronidase (Sigma type I), with 0.1 mM Ca^{2+}. A ratio of 10 ml of dissociation medium per g of pancreas was used. After injecting one half of the dissociation medium into the parenchyma, the pancreas was initially shaken in a polycarbonate flask at 120 cycles/min at 37°, for 10 min. At the end of this period, excess medium was removed, the remainder of the dissociation medium was added, and incubation was continued for 40–50 min. The rest of the isolation procedure was identical to that described by Williams *et al.* [16]. Unless indicated otherwise, isolated acini were incubated in a standard incubation medium (HRI) containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, 128 mM NaCl, 2.4 mM KCl, 1.13 mM $MgCl_2$, 1.0 mM sodium phosphate (pH 7.4), 1.28 mM $CaCl_2$, 14 mM glucose, 1% (w/v) bovine serum albumin (BSA), 0.01% (w/v) soybean trypsin inhibitor and minimal Eagle's amino acid supplement. Usually, isolated acini were preincubated for 60 min at 37° in a shaking water bath at 60 cycles/min, washed with fresh medium and resuspended, as detailed in the legends to figures and tables.

Amylase release and assay. Acini were suspended in incubation medium to a protein concentration of 0.5 to 1.0 mg/ml. At the beginning of the incubation period, 1.0 ml of the suspension was centrifuged at 10,000 rpm for 10 sec in an Eppendorf microcentrifuge. The pellet of acini was rinsed in ice-cold 0.9% NaCl, suspended in 1.0 ml of cold water and briefly sonicated to disrupt the acini. Amylase was assayed in the supernatant fraction and the sonicated acini, as detailed by Williams *et al.* [16], according to the procedure of Rinderknecht *et al.* [17], using Amylose azure dye (Sigma Chemical Co.). Amylase activity associated with the sonicated acini constituted the total amylase content at the beginning of a treatment. Under various conditions of incubation, acini were separated from the medium by microcentrifugation, for 10 sec, and supernatant fractions were preserved at 0–4°. Amylase released into the extracellular medium during incubation was calculated as the percentage of the total amylase activity present at the beginning of the experiment. In each experiment all assays were carried out in duplicate and results were averaged. Results are expressed either from a representative experiment or as means ± SEM of (N) values from several preparations of acini.

Lactic dehydrogenase (LDH). LDH was assayed according to the procedure of Farber and Young [18].

$^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake was measured as described by Dormer *et al.* [19]. Pancreatic acini (0.8 to 1.2 mg/ml) were incubated with $^{45}CaCl_2$ (2 μCi/ml, 1.28 mM Ca^{2+}). At predetermined intervals, 0.5-ml aliquots of the acini in duplicate were removed and diluted into 10 ml of ice-cold saline containing 1 mM $LaCl_3$. The acini were collected and washed on Nuclepore filters (3 μm pore size). The filters containing the cells were transferred to plastic tubes, 2 ml water was added, and acini were disrupted by sonication with the small probe of a Branson model 185 sonifier. Aliquots of the sonicate were dissolved in Budget-Solve liquid scintillation fluid (Res. Prod. International, IL) and counted in a Packard liquid scintillation counter. The amount of exchangeable calcium in the acini was determined on the basis of the specific activity of Ca^{2+} in the medium, and expressed as nmoles Ca^{2+}/mg protein.

$^{45}Ca^{2+}$ efflux. Acini (0.8 to 1.2 mg) were initially labeled with $^{45}CaCl_2$ (2 μCi/ml) for 60 min, centrifuged (200 *g* for 2 min), washed with fresh, ice-cold incubation medium, and resuspended in isotope-free medium at 37°. At time zero and subsequent specified times, 0.5-ml aliquots of acini in duplicate were removed and diluted with 10 ml of ice-cold saline. The acini that were collected on Nuclepore filters were processed as in "$^{45}Ca^{2+}$ uptake" experiments.

Determination of total cellular Ca^{2+} content. Total cellular Ca^{2+} was determined in pancreatic acini as described previously [20], using a Perkin–Elmer Atomic Absorption Spectrophotometer, model 460.

Determination of intracellular free Ca^{2+} using quin-2. Isolated pancreatic acini (3–4 mg protein/ml) were loaded with acetoxymethyl ester of quin-2(quin-2 AM, 60 μM final concentration) for 30 min at 37° in the HEPES-buffered incubation medium, in which the BSA content was reduced to 0.1% (w/v), as described by Oachs *et al.* [21] for mouse pancreatic acini. Briefly, after the acini were incubated with quin-2 AM for 30 min, they were diluted with an equal volume of the incubation medium and centrifuged (50 *g* for 2 min), washed once with the incubation medium equivalent to twice the original incubation volume, and finally resuspended in fresh incubation medium at a concentration of approximately 0.6 to 0.8 mg protein per ml and maintained at room temperature in oxygen atmosphere. Immediately before fluorescence recording, cells equivalent to 6–8 mg protein were centrifuged and suspended in 2 ml of the incubation medium maintained at 37°. The intracellular concentration of quin-2 did not exceed 0.5 mM. Fluorescence measurements were made in samples continuously stirred and maintained

at 37°, using a Perkin–Elmer spectrofluorimeter. The excitation and emission wavelengths were 339 and 492 nm respectively. Additions were made through a light-tight guide which did not affect monitoring. Unloaded cells exhibited negligible fluorescence under basal and stimulated conditions, and hence no corrections were made for the minor fluorescence changes observed in unloaded cells. Intracellular free Ca^{2+}, ($[Ca^{2+}]_i$), was determined from the relation described by Tsien *et al.* [22] as follows:

$$[Ca^{2+}]_i = K_d \frac{(F - F_{min})}{(F_{max} - F)}$$

where K_d is the apparent dissociation constant of quin-2 for calcium (115 nM); F is the fluorescence intensity in arbitrary units of intact, quin-2 loaded cells; F_{max} is the fluorescence intensity of quin-2 in the presence of saturating concentrations of calcium, as observed after addition of digitonin (50 μg/ml) to the cell suspension; and F_{min} is the fluorescence intensity of quin-2 in the absence of binding to Ca^{2+} [23] measured after addition of 10 mM ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA) with sufficient Tris to bring the final pH to 8.3.

Protein. Protein was estimated by the procedure of Lowry *et al.* [24], using BSA as the standard.

Electron paramagnetic resonance (EPR) measurements. Two types of spin labels were used to investigate possible alterations and localization of the probe in pancreatic membrane domains: (a) the order parameter probe, 12-doxyl stearic acid, which, when incorporated into acini, has its nitroxyl group positioned away from the aqueous phase, and buried in the hydrophobic interior of the membrane, and (b) TEMPO-stearic acid, which, when incorporated into acini, has its nitroxyl group proximal to the aqueous phase. Probes were purchased from Molecular Probes, Inc. (Junction City, OR). The probes were added to cell suspensions or isolated membrane systems from concentrated stock solutions (25 mM in ethanol) to a final concentration of approximately 2 nmoles/mg protein. The final concentration of ethanol in the medium after adding the probe did not exceed 17 mM.

Incorporation of the fatty acid spin probes into pancreatic acini. Isolated pancreatic acini were incubated in 4-ml suspensions, containing approximately 1 mg acinar protein/ml in the standard incubation medium in 25 ml polycarbonate Erlenmeyer flasks at 37°. Immediately before each treatment, acini were centrifuged (50 *g* for 2 min) and washed (50 *g* for 1 min) with 4 ml of incubation medium without BSA and finally suspended in 0.5 ml of the same medium in a 12 × 75 mm polystyrene test tube. A suitable concentration of the probe (in ethanol) was added in a volume of not more than 0.5 μl. This was followed by the addition of various concentrations of ethanol, mixed gently, and acini were allowed to settle for 3 min at room temperature. The concentrated suspension was transformed to a 100-μl capillary tube, using a 20-gauge stainless steel needle and plastic syringe. Using this procedure, there was no appreciable release of LDH for 30 min. Immediately after transferring the cells to the capillary, the sample was placed in the EPR cavity Dewar liner in a standard 4 mm quartz EPR tube, and scanned within a period of 5 min. EPR measurements were made at x-band microwave frequencies with a Varian E-109 B EPR spectrometer. Unless otherwise indicated, cell samples were analyzed at 37° using a Varian E-257 temperature controller. The EPR spectrometer was interfaced to a dedicated microcomputer, which allowed signal averaging and data analysis as described previously [11, 13]. In these studies the order parameter values were obtained using 12-doxyl stearic acid as the spin probe. EPR spectra were taken using the following instrumental settings: microwave power, 5 mW; modulation amplitude, 1.0 G; time constant, 0.128 sec; and scan time, 2 min. The order parameter, "*S*", for 12-doxyl stearic acid in acinar membranes was calculated from the inner ($T_\perp$) hyperfine splittings as described by Gaffney [25] using the relation

$$S = \frac{43.7\,G - 3\,T_\perp}{46.1\,G} \times 1.723$$

where $T_\perp$ is expressed in G and the constant 46.1 G depends on molecular parameters for the probe in the membrane [25, 26].

Statistics. All data presented were obtained from three or more independent experiments. Statistical significance was calculated by the paired *t*-test, using an HP-85 computer.

Materials. Secretagogue CCK_8 was a gift from Squibb Institute, Princeton, NJ; VIP was purchased from Peninsula Laboratories, Palo Alto, CA; divalent cation ionophore A23187 was obtained from Calbiochem, and other biochemical reagents from the Sigma Chemical Co. Quin-2 AM (acetoxymethyl ester of quin-2, see Ref. 22) was purchased from the Sigma Chemical Co., St. Louis, MO. $^{45}CaCl_2$ (1.34 Ci/mmole) was purchased from the Amersham Corp., Arlington Heights, IL.

RESULTS

Effects of ethanol on amylase secretion. Ethanol added *in vitro* at concentrations of 200 and 500 mM stimulated the release of amylase in isolated rat pancreatic acini. The effects of ethanol were transient, occurring during the first 10 min of exposure. Beyond 10 min, the rate of release was not significantly different from that of basal secretion over a period of 60 min (Fig. 1 and unpublished observations). However, at lower concentrations of ethanol, a longer (20 min) exposure time was necessary to observe a significant increase in amylase secretion. Under those conditions, a significant ($P < 0.05$) stimulation of secretion was obtained at ethanol concentrations of 50 mM and above (Fig. 2). No saturation of the effect of ethanol was apparent over the range of concentrations studied; rather, at concentrations of ethanol over 200 mM, amylase secretion was stimulated disproportionately, resulting in a somewhat sigmoidal dose–response relationship (Fig. 2). Cellular viability, as measured by LDH release, was in excess of 97% at all concentrations of ethanol (not shown). Ethanol alone, up to 500 mM, had no effect on the assay of amylase or LDH activities. In Table 1, the stimulation of pancreatic amylase secretion by ethanol is compared

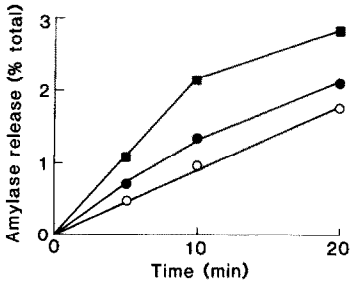

Fig. 1. Time-dependent effects of ethanol *in vitro* on amylase secretion in isolated rat pancreatic acini. Isolated acini were incubated in the presence of 200 and 500 mM ethanol. Amylase that was released into the medium over a period of 20 min was determined as detailed in Materials and Methods. Key: (○) control (●) 200 mM ethanol; and (■) 500 mM ethanol.

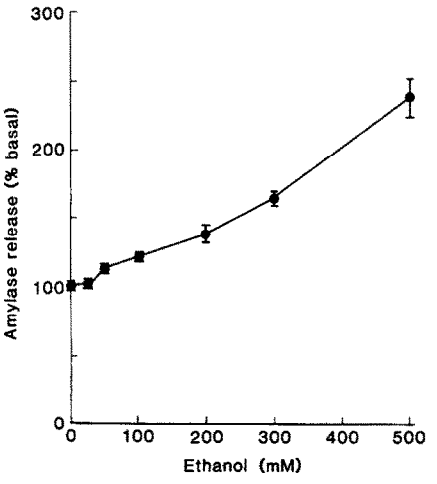

Fig. 2. Dose-dependent effects of ethanol *in vitro* on amylase secretion in isolated pancreatic acini. Isolated acini were incubated for 20 min in the presence of various concentrations of ethanol. Amylase that was released into the medium was determined as detailed in Materials and Methods. Ethanol-induced amylase secretion was statistically significant ($P < 0.005$–$P < 0.05$) at all concentrations of ethanol above 25 mM. Values presented are the mean ± SE obtained from duplicate analysis carried out with six separate preparations of acini using pancreases pooled from two rats per preparation.

with the effect of the pancreatic secretagogues, CCK_8 which stimulates secretion by a Ca^{2+}-mediated mechanism and VIP which operates via a cAMP-mediated pathway [27]. The concentrations of CCK_8 (500 pM) and VIP (10 nM) used were optimal for amylase secretion in isolated pancreatic acini, and were comparable to the levels used by others [8, 28]. In most of our experiments, an ethanol concentration of 200 mM was used. Although this concentration is higher than the circulating ethanol level in alcoholics [29], the effects of ethanol were qualitatively similar, but more pronounced than at lower concentrations. VIP stimulated secretion to a greater extent than ethanol, but was less potent than CCK_8. VIP and CCK_8, when added together, potentiated amylase secretion, in that the combined effect was in excess of the sum of the independent effects of the secretagogues. Similar synergistic actions of cAMP- and Ca^{2+}-mediated hormones have been reported by other groups [28, 30–32], but the extent of potentiation varied greatly. In our preparations of acini, we observed a considerable variation in the extent of secretion caused by VIP and that by VIP and CCK_8 added together, as indicated by high standard errors (Table 1). However, in all preparations, the effect of ethanol was qualitatively similar and consistent. Paired statistical analysis indicated that the stimulation of amylase secretion by the combinations of ethanol and VIP was more than additive compared to the stimulation by these agents separately ($P < 0.005$); thus ethanol also had a potentiating effect on VIP-induced secretion. By contrast, ethanol had little effect on secretion induced by CCK_8 alone or by the combination of CCK_8 and VIP. As with CCK_8, ethanol had no effect on the stimulation of secretion by carbachol, which also acts by a Ca^{2+}-mediated mechanism (data not shown). Thus, ethanol has a differential effect on Ca^{2+}-mediated and cAMP-mediated pathways of stimulation of amylase secretion.

Effects of ethanol on calcium homeostasis. Calcium-mediated secretagogues, such as CCK_8, are thought to mobilize intracellular calcium stores, thus

Table 1. Effect of ethanol *in vitro* on basal and secretagogue-stimulated amylase secretion in isolated pancreatic acini

		Amylase release (% of total)		
Additions	N	Control	Ethanol (200 mM)	Ethanol-induced change
None	6	1.3 ± 0.25	1.7 ± 0.33	0.4 ± 0.12*
VIP (10 nM)	6	3.6 ± 0.80	4.6 ± 0.95	1.0 ± 0.17†
CCK_8 (500 pM)	5	10.4 ± 1.4	10.3 ± 1.4	NS‡
VIP + CCK_8	5	20.3 ± 4.7	18.9 ± 3.7	NS

Isolated acini were incubated for 20 min under standard conditions with secretagogues as indicated, in the presence or absence of 200 mM ethanol. Values are means ± SE of (N) experiments.

* $P < 0.02$.

† $P < 0.005$.

‡ Not significant.

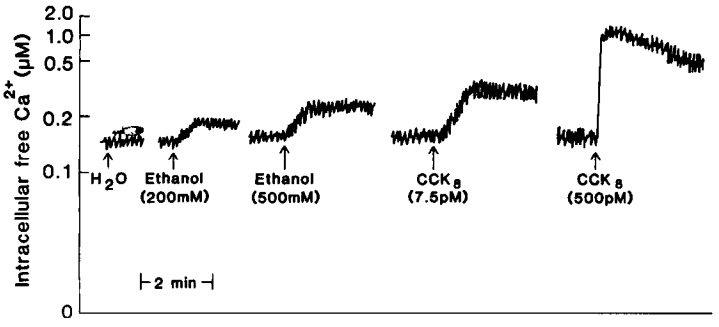

Fig. 3. Effects of ethanol and CCK_8 on intracellular free Ca^{2+} in isolated pancreatic acini. Acini loaded with quin-2 were exposed to ethanol (200 and 500 mM) and CCK_8 (7.5 pM and 500 pM). Figure indicates representative tracings under various conditions of treatment. Further details on quin-2 loading and determination of intracellular free Ca^{2+} are described in Materials and Methods.

leading to an increase in cytosolic Ca^{2+} levels. Pancreatic acini loaded with the calcium indicator quin-2 [21] were used to detect changes in cytosolic free Ca^{2+} in response to different secretagogues. A small, but significant ($P < 0.05$), increase in intracellular free Ca^{2+} was detected at 200 mM ethanol, and this effect was more pronounced at 500 mM ethanol (Figs. 3 and 4). The concentration of quin-2 accumulated in the acini (approximately 0.5 mM) can introduce a significant calcium-buffering capacity (see, for instance, Ref. 9) which can suppress the free Ca^{2+} concentration at the peak of the agonist response. The peak cytosolic free Ca^{2+} levels shown in Figs. 3 and 4 should, therefore, be considered minimum estimates of the free Ca^{2+} level induced by ethanol in unloaded acini. A dose-dependent increase in cytosolic free Ca^{2+} by CCK_8 and other secretagogues has been reported for mouse and guinea pig pancreatic acini [21, 33, 34]. We found a similar response in rat pancreatic acini. CCK_8, at a concentration (7.5 pM) which submaximally increased amylase secretion, also increased the cytosolic free Ca^{2+} concentration (Fig. 3). In the presence of this submaximal level of CCK_8, ethanol had no further effect on intracellular free Ca^{2+} (Fig. 4). VIP (10 nM) alone did not affect intracellular free Ca^{2+}, nor did it alter the changes in cytosolic free Ca^{2+} induced by ethanol or CCK_8 (data not shown). In other control experiments, ethanol *per se* had no effect on the fluorescence yield of the quin 2–Ca^{2+} complex. The lack of effect of ethanol (200 mM) on the level of cytosolic Ca^{2+} in the CCK_8-stimulated acini was paralleled by a lack of effect on amylase secretion (Fig. 4). However, at 500 mM, ethanol stimulated amylase release, even in the presence of a suboptimal CCK_8 concentration, despite the lack of a corresponding change in cytosolic Ca^{2+} level. It is also evident from Fig. 4 that 500 mM ethanol caused more stimulation of amylase release than 7.5 pM CCK_8, although its effect on cytosolic free Ca^{2+} was significantly lower.

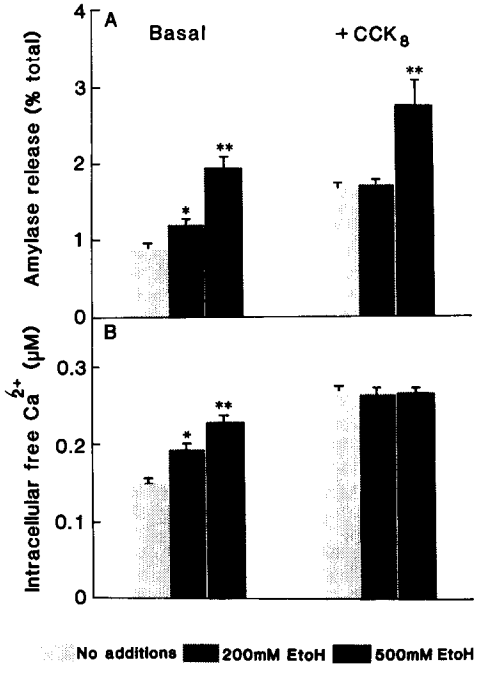

Fig. 4. Effects of ethanol and sub-maximal CCK_8 on amylase release and intracellular free Ca^{2+} in isolated pancreatic acini. Acini were incubated in the presence of 200 and 500 mM ethanol with and without 7.5 pM CCK_8. (A) Amylase that was released into the medium during a 10-min incubation period was determined as described in Materials and Methods. (B) Acini loaded with quin-2 were treated with 200 and 500 mM ethanol in the presence or absence of 7.5 pM CCK_8. Intracellular, free Ca^{2+} levels were determined as detailed in Materials and Methods. Values are means ± S.E. of four to five determinations using averages of duplicates from a preparation of acini per determination. Key: (*) $P < 0.05$; and (**) $P < 0.02$ compared to controls in the absence of ethanol.

We also studied the effects of various concentrations of ethanol on cellular calcium fluxes by measuring $^{45}Ca^{2+}$ uptake and efflux (Figs. 5 and 6). There was a small dose-dependent increase in the rate of both $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux by ethanol in isolated acini, at concentrations of 200 mM and above. Addition of CCK_8 greatly increased the rate of $^{45}Ca^{2+}$ efflux, in agreement with published data [20, 35–37]. The addition of ethanol (200 and 300 mM) also stimulated the initial rate of $^{45}Ca^{2+}$ efflux to a small extent, but had no effect on the rate of $^{45}Ca^{2+}$ exchange after 10 min. No effect of ethanol on $^{45}Ca^{2+}$ exchange was apparent in acini stimulated with CCK_8 (500 pM) (Fig. 6). Other studies [19, 20]

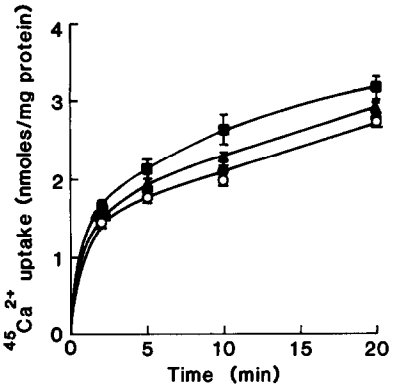

Fig. 5. Effect of ethanol on *in vitro* $^{45}Ca^{2+}$ uptake in isolated pancreatic acini. Isolated pancreatic acini were incubated with $^{45}CaCl_2$ (2 μCi/ml) and various concentrations of ethanol over a period of 20 min. $^{45}Ca^{2+}$ that was retained by the acini was determined as described in Materials and Methods. Key: (○) control; (●) 100 mM ethanol; (▲) 200 mM ethanol; and (■) 300 mM ethanol. Points are the means ± SE (bars) from three determinations using averages of duplicate values from a preparation per determination.

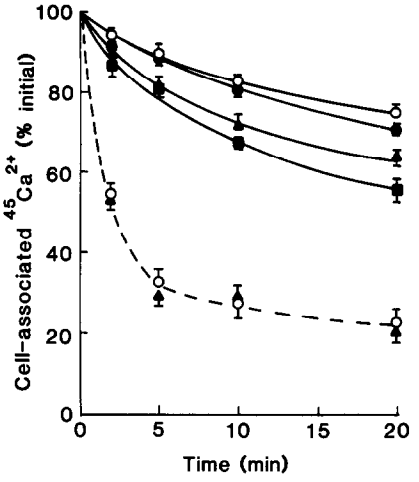

Fig. 6. Effect of ethanol *in vitro* on $^{45}Ca^{2+}$ efflux from isolated pancreatic acini. Acini were initially incubated with $^{45}CaCl_2$ (2 μCi/ml) for 60 min, washed, and resuspended in fresh medium in the absence of the isotope. The rate of efflux of $^{45}Ca^{2+}$ from the acini in the presence of various concentrations of ethanol and CCK_8 (500 pM) was determined as detailed in Materials and Methods. Key: (○) control; (●) 100 mM ethanol; (▲) 200 mM ethanol; (■) 300 mM ethanol. Broken lines indicate presence of CCK_8. Points are the means ± SE of samples from three separate preparations of acini.

have demonstrated that CCK_8 brings about a net efflux of calcium from the acini, presumably as a consequence of an increased cytosolic Ca^{2+} level. The present data suggest that ethanol may mimic to some extent these effects of CCK_8. The small degree of ethanol-induced change in $^{45}Ca^{2+}$ flux was compatible with the relatively modest effect of ethanol on the intracellular free Ca^{2+} concentration.

Effect of removal of extracellular Ca^{2+} *on ethanol-induced amylase secretion and* Ca^{2+} *mobilization.* When acini were suspended in HRI medium without added Ca^{2+}, but containing 0.1 mM EGTA, and incubated for 10 min at 37°, amylase release was not decreased substantially. Under those conditions, stimulation of amylase release by ethanol was inhibited completely at 200 mM ethanol, whereas significant stimulation was perceptible at 500 mM (Table 2). Again, under similar conditions, using quin-2 loaded acini, the absence of extracellular Ca^{2+} caused a decrease in the steady-state free Ca^{2+} level under basal conditions, and inhibited the rise in intracellular free Ca^{2+} induced by 200 mM ethanol; that by 500 mM ethanol still had a small, but significant effect on cytosolic Ca^{2+} levels. It is unlikely, however, that this small increase in free Ca^{2+} level is by itself sufficient to account for the stimulation of amylase secretion. These results suggest that, at lower concentrations of ethanol, ethanol-induced

Table 2. Effect of removal of extracellular Ca^{2+} on ethanol-induced amylase secretion and rise in intracellular free Ca^{2+} in isolated pancreatic acini

Additions	Amylase release (% of total)	Intracellular free Ca^{2+} (μM)
None	0.99 ± 0.03 (4)	0.081 ± 0.007 (6)
200 mM Ethanol	0.94 ± 0.02 (3)	0.080 ± 0.005 (4)
500 mM Ethanol	1.79 ± 0.09* (4)	0.113 ± 0.009† (6)

Isolated acini were suspended in HRI medium containing no added Ca^{2+}. EGTA (0.1 mM final concentration) was added 20 sec before each treatment, and amylase released during 10 min of incubation (37°) was determined as described in Materials and Methods. For the determination of intracellular free Ca^{2+}, acini loaded with quin-2 were suspended in HRI medium containing no added Ca^{2+} and 0.1% BSA. EGTA (0.1 mM final concentration), was added as above. Intracellular free Ca^{2+} concentrations were determined as detailed in Materials and Methods. All values are the mean ± SE of (N) preparations of acini using the average of duplicate determinations per treatment per preparation. (See Fig. 4 for the effects of ethanol on amylase release and intracellular free Ca^{2+} in the presence of 1.28 mM Ca^{2+}).

* $P < 0.01$, compared to basal conditions.

† $P < 0.02$, compared to basal conditions.

Table 3. Effect of chronic ethanol feeding on the sensitivity of basal and carbachol-stimulated amylase secretion to ethanol treatment *in vitro* in isolated pancreatic acini

Treatment	Additions	Amylase release			
		Control		Ethanol-fed	
		% of Total	% Change	% of Total	% Change
Basal	None (N = 5)	1.68 ± 0.1	0	1.15 ± 0.16*	0
	Ethanol (N = 3)	2.15 ± 0.1	24 ± 2	2.10 ± 0.28	109 ± 7†
Carbachol (10 μM)	None (N = 3)	7.36 ± 1.0	0	8.79 ± 0.80	0
	Ethanol (N = 3)	7.20 ± 0.5	−6 ± 2	8.24 ± 0.72	0 ± 7

Isolated pancreatic acini were prepared from ethanol-fed rats and pair-fed controls as described in Materials and Methods. Amylase released over a 20-min incubation period in the presence or absence of 200 mM ethanol was determined. Values are means ± SE of (N) pairs.

* $P < 0.01$ for amylase release between control and ethanol-fed groups.

† $P < 0.05$ for ethanol-induced change in amylase release between the two groups.

amylase secretion is dependent on extracellular Ca^{2+}; at higher concentrations, ethanol-induced amylase secretion may occur by other mechanisms in addition to that mediated by Ca^{2+}.

Effect of chronic ethanol consumption on amylase secretion in vitro. Chronic ethanol ingestion caused a 15–30% reduction in the basal rate of amylase secretion in isolated pancreatic acini (Table 3, Fig. 7). The addition of ethanol stimulated basal amylase secretion both in control preparations and in acini from ethanol-fed animals, but the extent of stimulation was greater in acini from the ethanol-fed rats, thus compensating for the decrease in the basal rate of secretion (Table 3, Fig. 8). The rate of CCK_8-stimulated secretion was slightly higher in acini from the ethanol-fed group (Fig. 7). Addition of ethanol (200 mM) to acini from either the control or the ethanol-fed group had no significant effect on amylase secretion by the Ca^{2+}-mediated secretagogue, carbachol (Table 3).

Effects of chronic ethanol consumption on calcium homeostasis. The difference in amylase secretion between preparations from the control and ethanol-fed rats was not associated with a difference in cytosolic free Ca^{2+}. As shown in Table 4, using acini loaded with quin-2, under steady-state conditions, the level of intracellular free calcium was not changed by chronic ethanol feeding. When ethanol was added *in vitro*, the increase in the level of intracellular free Ca^{2+} concentration was similar in acini from control and ethanol-fed groups (Table 4).

The rate of $^{45}Ca^{2+}$ uptake (which reflects calcium exchange with the extracellular medium) was decreased significantly in acini from the ethanol-fed rats (Fig. 9). This difference was detectable both in

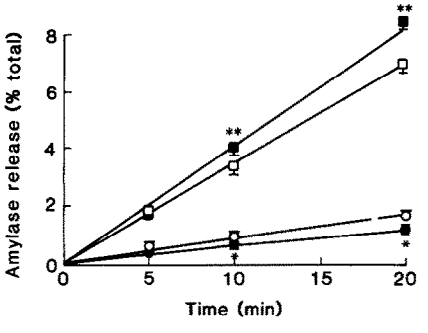

Fig. 7. Effect of chronic ethanol feeding on the rate of amylase secretion in isolated pancreatic acini. Acini from control (○, □) and ethanol-fed (●, ■) animals were incubated in the presence (□, ■) or absence (○, ●) of 500 pM CCK_8. Amylase released at various intervals of time was determined as described in Materials and Methods. Points are the means ± SE of seven preparations of acini. Key: (*) $P < 0.05$, and (**) $P < 0.005$ between ethanol-fed and pair-fed control groups.

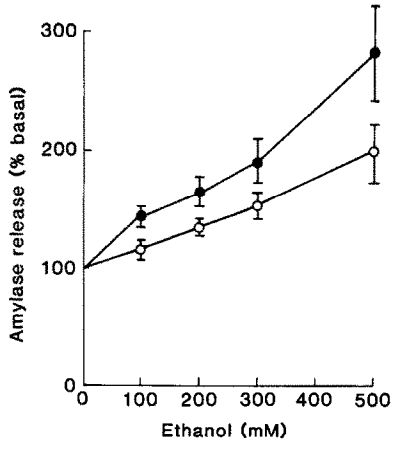

Fig. 8. Effect of the addition of ethanol *in vitro* on the basal rate of amylase secretion in acini from control and ethanol-fed rats. Isolated acini from control (○) and ethanol-fed (●) rats were incubated for 20 min in the presence of various concentrations of ethanol. Amylase that was released into the medium was determined as described in Materials and Methods. Amylase secretion by both the groups in the presence of ethanol is expressed as percent of the respective basal amylase that was secreted in the absence of ethanol. Each point is the average of values from four to six determinations, using a pair of rats per determination. Ethanol-induced amylase secretion was significant at all concentrations of ethanol (also see Fig. 2). The extent of ethanol-induced amylase secretion by acini from the ethanol-fed group was greater ($P < 0.02$ to $P < 0.05$) than that in the control group (also see Table 3).

Table 4. Effects of chronic ethanol-feeding and of ethanol *in vitro* on intracellular free Ca^{2+} concentration in pancreatic acini

Additions	Intracellular free Ca^{2+} (μM)	
	Control	Ethanol-fed
None	0.155 ± 0.012 (7)	0.143 ± 0.009 (7)
200 mM ethanol	0.195 ± 0.019 (6)	0.185 ± 0.022 (6)
500 mM ethanol	0.234 ± 0.020 (6)	0.229 ± 0.030 (6)

The levels of intracellular free Ca^{2+} were determined in acini loaded with quin-2 as described in Materials and Methods. All values are the mean ± SE of (N) determinations. Separate pairs of animals were used for each determination. Paired *t* analysis showed that in the same group each of the treatments induced changes that were statistically different ($P < 0.02$ to 0.05) from each other. There was no statistical difference in the levels of intracellular free Ca^{2+} concentration between control and ethanol-fed groups either in the absence or presence of ethanol.

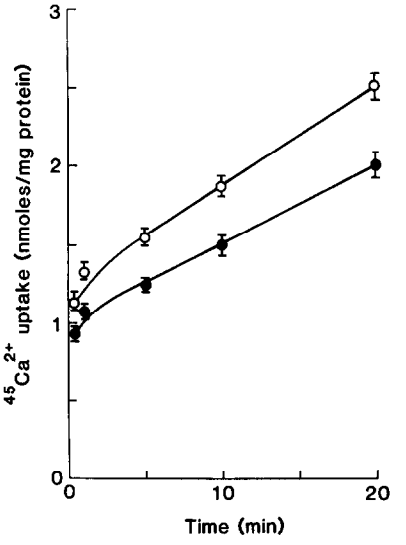

Fig. 9. Effect of chronic ethanol feeding on the rate of $^{45}Ca^{2+}$ uptake by pancreatic acini. Isolated acini from control (○) and ethanol-fed (●) rats were incubated with $^{45}CaCl_2$ (2 μCi/ml) in HRI medium. The amount of $^{45}Ca^{2+}$ that was retained by the acini at various intervals of time was determined as described in Materials and Methods. Each point is the mean ± SE (bars) of four determinations, using acini from a pair of rats per determination. $P < 0.05$ at 20 sec, 1 min and 5 min; $P < 0.01$ at 10 min and $P < 0.005$ at 20 min between the two groups.

the initial rapid exchange (at t = 20 sec) and in the secondary slower phase of $^{45}Ca^{2+}$ uptake. Total cellular Ca^{2+} content was also lowered by 11% (Table 5). This difference in intracellular calcium content was additionally reflected in the amount of calcium that could be mobilized by the ionophore A23187. In acini from the ethanol-fed group, the amount of mobilizable calcium, was about 24% lower than in the control group.

Effects of in vitro *and chronic ethanol treatment on membrane molecular order in isolated rat acinar preparations.* Ethanol affects the organization of cellular membranes both *in vitro* and after chronic ethanol treatment [11–14]. Therefore, we investigated whether the effects of ethanol on the metabolic activities in pancreatic acini would correlate with changes in membrane organization. Studies were carried out with spin-labeled stearic acid derivatives incorporated into the membranes of pancreatic acini. No appreciable reduction of the spin-probe occurred when intact acini were labeled with either 12-doxyl stearate or TEMPO stearate. Spectra were stable well beyond the period of 2 min required to run the experiment. When the acini were mechanically disrupted, the probe signal was rapidly lost by reduction, a finding which indicates that in the intact cell little or no probe was accessible to the reducing processes in the interior of the cell. Rapid and complete probe reduction occurred when acini labeled with TEMPO stearate were incubated with 20 mM ascorbate at 37°. A similar reduction of the probe occurred when acini labeled with TEMPO-stearate were incubated with ascorbate at 2°, so as to prevent any remaining permeation of ascorbate across the plasma membrane. These results indicate that the stearic acid derivatives were freely accessible to external reductants, but not to intracellular reducing systems. Thus, during the time of the experiment, the spin probes appeared to be localized predominantly in the outer face of the plasma membrane.

Table 5. Effect of chronic ethanol feeding on cellular calcium content in pancreatic acini

Treatment	Total Ca^{2+} content	Ca^{2+} released by A23187
	(nmoles/mg protein)	
Control	12.22 ± 0.79	2.51 ± 0.20
Ethanol-fed	10.91 ± 0.57*	1.92 ± 0.07†

Total cellular calcium content in acini was determined as described in Materials and Methods. Values are the mean ± SE of five pairs. The amounts of Ca^{2+} released by the ionophore A23187 (5 μM, 37°, 30 min) were 20.5 ± 1.1 and 17.6 ± 0.8 (expressed as percent of total calcium content) for control and ethanol-fed groups, respectively, and were not statistically different from each other.

* $P < 0.01$ between control and ethanol-fed groups.

† $P < 0.05$ between control and ethanol-fed groups.

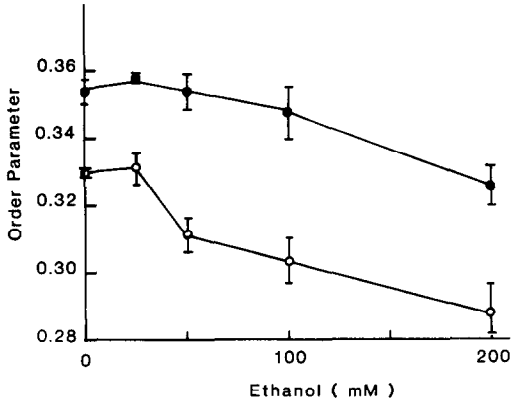

Fig. 10. Effects of acute and chronic ethanol feeding on membrane order parameters in isolated rat pancreatic acini. Order parameters were calculated from EPR spectra of acini labeled with 12-doxyl stearic acid as detailed in Materials and Methods, in the presence of various concentrations of ethanol. Ethanol concentration of the probe-solution was taken into account in calculating the final concentration of ethanol. Each point is the mean ± SE (bar) of three pairs of determinations, In control (○) acini, ethanol at concentrations of 50 mM and above significantly ($P < 0.05$) fluidized acinar membrane. In acini from the ethanol-fed (●) group, significant ($P < 0.05$) fluidization occurred only at 200 mM ethanol. The order parameter values were statistically significant ($P < 0.05$) between control and ethanol-fed groups under all conditions of treatment.

Subcellular fractions, isolated from intact acini by differential centrifugation and labeled with 12-doxyl stearate, displayed characteristic values for the order parameter. The order parameters were as follows: mitochondria, 0.370; microsomes, 0.288; plasma membranes, 0.330; and intact acini, 0.328. The post-microsomal supernatant fraction rapidly reduced the probe. The similarity in the order parameter between the fraction enriched in plasma membranes and intact acini further supports the interpretation that the spin probes resided in the plasma membranes of intact acini. Our conclusion that the spin probes are predominantly located in the plasma membrane is in agreement with the results of Kaplan *et al.* [38], who used spin probes in intact mammalian cells, such as human lymphocytes, mouse L-cells and erythrocytes.

In control animals, significant fluidization of acinar membranes occurred at ethanol concentrations of 50 mM and above (Fig. 10); similar data were obtained with preparations from chow-fed animals (not shown). Chronic ethanol ingestion caused an increase in the order parameter of acinar membranes; $S = 0.353 \pm 0.005$ for ethanol-fed animals vs 0.329 ± 0.002 for controls (mean ± SE for four pairs, using 12-doxyl stearic acid as the spin probe). Acinar cell membranes from ethanol-fed rats showed greater resistance to the disordering effects of ethanol added *in vitro* than those from pair-fed controls (Fig. 10). In the ethanol-fed groups, significant changes in order parameter occurred only at an ethanol concentration of 200 mM.

DISCUSSION

The experimental work reported in this paper forms part of a large study aimed at understanding the consequences of ethanol–membrane interactions for cellular metabolism. In studies on isolated hepatocytes [9], we reported a marked transient disturbance of intracellular calcium homeostasis by ethanol, possibly mediated through an activation of the hormone-sensitive pathway of polyphosphoinositide metabolism. Since the same pathway is also involved in stimulus–secretion coupling in pancreatic acini, a similar action of ethanol might occur.

Earlier reports [7, 8] indicated that ethanol, in high concentrations (> 0.4 M), inhibits the effect of CCK_8 and other secretagogues on amylase secretion in guinea pig pancreatic acinar cells. The data reported here indicate that, in rat acini, ethanol in concentrations of 50 mM and above acts as a weak secretagogue. Several lines of evidence suggest that an elevation of the cytosolic free calcium concentration is an essential component of this response.

In the first place, ethanol causes a concentration-dependent increase in cytosolic free calcium levels. Second, supportive evidence was obtained from a study of the combined effects of ethanol and hormones that activate the two branches of the secretory trigger system, namely, CCK_8 (Ca^{2+}-mediated) and VIP (cAMP-mediated). The two messenger systems are not independent, as evidenced by their synergistic action on amylase secretion [28–32]. Ethanol significantly potentiated the action of VIP ($P < 0.005$), but it had no effect on the amylase release induced by optimal concentrations of CCK_8. This suggests that ethanol mimics the action of calcium-mobilizing hormones.

However, the data also provide indications that factors other than calcium may be involved in the response to ethanol. Theoretically, the combined effects of submaximal concentrations of two different secretagogues, operating through the same pool of intracellular calcium, should be additive. The effects of a high concentration (500 mM) of ethanol and a low concentration of CCK_8 on amylase secretion were indeed additive. However, this effect was not associated with an additive effect in the levels of cytosolic free calcium (see Fig. 4). Moreover, at high concentrations of ethanol the burst in amylase secretion was not directly proportional to the rise in intracellular free calcium, a result which indicates that other mechanisms may contribute to its effect, such as a rise in cAMP. A small but a significant increase in cAMP at high concentration (0.6 M) of ethanol was reported by Uhlemann *et al.* [7] in guinea pig acini. We have attempted to find evidence for this possibility but were not successful, due to the extreme variability of cAMP assays in this tissue (compare also Ref. 28). It should also be pointed out that ethanol (500 mM) and CCK_8 may not act as independent secretagogues, because high concentrations of ethanol have been reported to inhibit CCK_8 binding to mouse pancreatic acini [8].

In contrast to the effects of high levels of ethanol, lower (up to 200 mM) ethanol concentrations appeared to affect the acini exclusively by a calcium-

mediated mechanism. Extracellular calcium was an absolute requirement for the stimulation of amylase secretion by 200 mM ethanol and, also, the rise in intracellular calcium was inhibited completely in the absence of extracellular calcium. The decrease in cytosolic free calcium levels, after removal of extracellular calcium from quin 2-loaded cells, suggests the possibility that there was some depletion of intracellular calcium stores as a consequence of the washing procedure.

Changes in cellular calcium homeostasis after chronic ethanol treatment of the rat were evident in the isolated pancreatic acini, both by a decrease in the total and ionophore-mobilizable pool of calcium and by a decrease in the rate of exchange of extracellular $^{45}Ca^{2+}$ with intracellular calcium. Interestingly, this was not accompanied by a significant change in the cytosolic free Ca^{2+} concentration. The low rate of $^{45}Ca^{2+}$ uptake in acini from the ethanol-fed group is difficult to interpret. It could be due to a difference in the rate of calcium exchange across the plasma membrane or to a slower equilibration of cytosolic Ca^{2+} with internal calcium storage or binding sites. Alternatively, it could be a reflection of a difference in pool size of intracellular calcium stores. The latter interpretation is supported by the observation that the rate of $^{45}Ca^{2+}$ uptake was decreased after chronic ethanol feeding to the same extent as the total and ionophore-mobilizable pool of calcium in the acini. In fact, there was no significant difference between the two preparations if the rate of $^{45}Ca^{2+}$ uptake were expressed as a fraction of the mobilizable pool of calcium. These data suggest that the predominant differences in the calcium status of pancreatic acini from ethanol-fed and control rats is in the pool sizes of intracellular stores.

Cytosolic calcium levels were very similar in the acini from control and ethanol-fed rats, both in untreated acini and in the presence of ethanol (Table 4). However, the basal rate of amylase release in the acini from ethanol-fed rats was significantly lower than in the control preparations. This difference disappeared when the acini were stimulated with ethanol or a calcium-mediated secretagogue, resulting in a significantly higher percentage stimulation by these agents (Table 3 and Fig. 8). Thus, the decrease in cellular calcium stores by ethanol feeding did not impair the capacity of the system to respond to physiological stimuli. The results indicate that the sensitivity of the secretory systems to changes in the cytosolic calcium level is different in the acini from ethanol-fed and control rats. The mechanism(s) responsible for regulating the sensitivity of these responses has not been clarified, but it is conceivable that membrane structural parameters could play a role in this respect. It should be pointed out that in a recent report [39] we observed no apparent changes in the number of zymogen granules in acini from rats after long-term ethanol ingestion, to account for the decreased rate of amylase secretion.

Isolated pancreatic acinar preparations exhibit disordering effects of ethanol similar to other membrane preparations obtained from various tissues [11–14]. Our data suggest that, in the acinar preparations labeled with spin probes, the EPR signal derives from the plasma membrane. This conclusion is in agreement with other studies, using fatty acid spin labels in intact cells [38, 40]. We cannot distinguish between the basolateral and apical membranes of the acini. It is likely, however, that the effects of ethanol are also exerted equally on other intracellular membranes that do not contribute to the EPR signal. The acinar membranes from ethanol-fed rats are less susceptible to the disordering effect of ethanol than those from pair-fed controls, similar to observations with other subcellular membrane systems [11–14]. Thus, the ethanol-induced difference in membrane organization can also be detected in intact cells.

The data reported here do not support the interpretation that the effects of ethanol on amylase secretion and calcium homeostasis are a direct consequence of the changes in membrane molecular ordering induced by ethanol. In acini from rats fed a control diet (which behaved identically to those from chow-fed rats in this respect), a threshold concentration of 50 mM ethanol induced a large change in the membrane order parameter. Changes in amylase secretion were barely significant at this concentration of ethanol, and higher concentrations were required to cause a detectable increase in the cytosolic free Ca^{2+} level. Moreover, acini from ethanol-fed rats were resistant to the disordering effects of these concentrations of ethanol; by contrast, the ethanol-induced increase of the cytosolic free Ca^{2+} concentration was unchanged in acini from ethanol-fed rats, and amylase secretion was stimulated even more than in control cells.

The experimental data on ethanol-fed animals reported here were obtained with preparations that do not exhibit the morphological evidence of cellular damage, as reported after longer term (> 6 months) ethanol treatment [2]. It is possible, however, that the effects of ethanol ingestion on membrane structural characteristics and on cellular calcium homeostasis are early responses of the pancreatic acini to the presence of ethanol, which may make the cells more susceptible to damage, either after longer exposure or in response to other toxins [41].

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REFERENCES

1. H. Sarles and R. Laugier, *Clin. Gastroent.* **10**, 402 (1981).
2. N. Darle, R. Ekholm and Y. Edlund, *Gastroenterology* **58**, 62 (1970).
3. R. Huttunen, P. Huttunen and P. Jalovaara, *Scan. J. Gastroent,* **11**, 103 (1975).
4. S. Matsuno, K. Kano, K. Miyagawa, H. Yamaguchi and T. Sato, *Tohoku J. exp. Med.* **141**, 77 (1983).
5. M. Noronha, A. Salgadinho, M. J. Ferreira de Almeida, D. A. Dreiling and O. Bordalo, *Am. J. Gastroent.* **76**, 114 (1981).
6. E. Rubin, in *The Liver* (Ed. E. A. Gall), p. 199. William & Wilkins, Baltimore, MD (1972).

7. E. R. Uhlemann, P. Robberect and J. D. Gardner, *Gastroenterology* **76**, 917 (1979).
8. M. B. Levin, H. Sankaran, C. W. Deveney, A. Wong, M. F. Wendland and M. C. Geokas, *Biochem. Pharmac.* **33**, 3225 (1984).
9. J. B. Hoek, A. P. Thomas, R. Rubin and E. Rubin, *J. biol. Chem.* **262**, in press.
10. F. Clemente, S. Durand, J. Laval, J. P. Thouvenot and A. Ribet, *Gastroent. Clin. Biol.* **1**, 39 (1976).
11. H. Rottenberg, A. J. Waring and E. Rubin, *Science* **213**, 583 (1981).
12. A. J. Waring, H. Rottenberg, T. Ohnishi and E. Rubin, *Proc. natn. Acad. Sci. U.S.A.* **78**, 2582 (1982).
13. A. J. Waring, H. Rottenberg, T. Ohnishi and E. Rubin, *Archs Biochem. Biophys.* **216**, 51 (1982).
14. B. C. Ponnappa, A. J. Waring, J. B. Hoek, H. Rottenberg and E. Rubin, *J. biol. Chem. 257*, 10141 (1982).
15. L. M. DeCarli and C. S. Lieber, *J. Nutr.* **91**, 331 (1967).
16. J. A. Williams, M. Korc and R. L. Dormer, *Am. J. Physiol.* **235** (Endocrin. Metab. Gastrointest. Physiol. 4), E517 (1978).
17. J. Rinderknecht, P. Wilding and B. J. Haverback, *Experientia* **23**, 805 (1967).
18. J. L. Farber and E. A. Young, *Archs. Biochem. Biophys.* **211**, 312 (1981).
19. R. L. Dormer, J. H. Poulsen and J. A. Williams, *Am. J. Physiol.* **240** (Gastrointest. Liver Physiol. 3), G38 (1981).
20. B. C. Ponnappa and J. A. Williams, *Cell Calcium* **1**, 267 (1981).
21. D. L. Oachs, J. I. Korenbrot and J. A. Williams, *Biochem. biophys. Res. Commun.* **117**, 122 (1983).
22. R. Y. Tsien, T. Pozzan and T. J. Rink, *J. Cell Biol.* **94**, 325 (1982).
23. T. J. Halloran, A. Sanchez and T. J. Rink, *Biochem. J.* **218**, 819 (1984).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. B. J. Gaffney, *Proc. natn. Acad. Sci. U.S.A.* **72**, 664 (1975).
26. B. L. Bales, E. S. Lesin and S. B. Oppenheimer, *Biochim. biophys. Acta* **465**, 400 (1977).
27. J. D. Gardner, *A. Rev. Physiol.* **41**, 55 (1979).
28. M. J. Collen, V. E. Stuliff, G-Z. Pan and J. D. Gardner, *Am. J. Physiol.* **242** (Gastrointest. Liver Physiol. 5), G423 (1982).
29. E. Rubin and H. Rottenberg, *Fedn. Proc.* **41**, 2465 (1982).
30. M. Descholt-Lanckman, P. Robberect, P. DeNeef, F. Labrie and J. Christophe, *Gastroenterology* **68**, 318 (1975).
31. M. Singh, *J. Physiol., Lond.* **331**, 547 (1982).
32. D. B. Burnham, D. J. McChesney, K. C. Thurston and J. A. Williams, *J. Physiol., Lond.* **349**, 475 (1984).
33. R. E. Powers, P. C. Johnson, M. J. Houlihan, A. K. Saluja and M. L. Steer, *Am. J. Physiol.* **248**, (Cell Physiol. 17), C535 (1985).
34. S. J. Pandol, M. S. Schoeffield, G. Sachs and S. Mualleem, *J. biol. Chem.* **260**, 10081 (1985).
35. I. Schulz and H. Stolze, *A. Rev. Physiol.* **42**, 127 (1980).
36. I. Schulz, *Am. J. Physiol.* **239**, (Gastrointest. Liver Physiol. 2), G335 (1980).
37. J. A. Williams, *Am. J. Physiol.* **238** (Gastrointest. Liver Physiol. 1), G269 (1980).
38. J. Kaplan, P. Caronito and W. Caspary, *Proc. natn. Acad. Sci. U.S.A.* **70**, 66 (1973).
39. B. C. Ponnappa, J. B. Hoek, K. Sarchet and E. Rubin, *J. Lab. clin. Med.* **107**, 556 (1986).
40. A. Aszalos, G. C. Yang and M. M. Grottesman, *J. Cell Biol.* **100**, 1357 (1985).
41. F.A.X. Schanne, A. H. Zucker, J. L. Farber and E. Rubin, *Science* **212**, 338 (1981).