Interaction of Ethanol with Inducers of Glucose-regulated Stress Proteins

ETHANOL POTENTIATES INDUCERS OF grp78 TRANSCRIPTION*

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GRP78, a molecular chaperone expressed in the endoplasmic reticulum, is a “glucose-regulated protein” induced by stress responses that deplete glucose or intracisternal calcium or otherwise disrupt glycoprotein trafficking. Previously we showed that chronic ethanol exposure increases the expression of GRP78. To further understand the mechanism underlying ethanol regulation of GRP78 expression, we studied the interaction between ethanol and classical modulators of GRP78 expression in NG108-15 neuroblastoma x glioma cells. We found that, in addition to increasing basal levels of GRP78 mRNA ("induction"), ethanol produced greater than additive increases in the induction of GRP78 mRNA by the "classical" GRP inducers A23187, brefeldin A, and thapsigargin ("potentiation"). Both the ethanol induction and potentiation responses modulated grp78 gene transcription as determined by stable transfection analyses with the rat grp78 promoter. Ethanol potentiated the action of all classical inducers of grp78 transcription that were studied. In contrast, co-treatment with the classical GRP inducers thapsigargin and tunicamycin produced only simple additive increases in grp78 promoter activity. Transient transfection studies with deletion mutants of the rat grp78 promoter showed that cis-acting promoter sequences required for ethanol induction differ from those mediating responses to classical GRP inducers. Furthermore, linker-scanning mutations of the grp78 promoter suggested that the ethanol potentiation response required a cis-acting promoter element different from those involved in induction by ethanol or classical inducing agents. While the ethanol induction response required 16–24 h to be detectable, ethanol potentiation of thapsigargin occurred within 6 h. The potentiation response also decayed rapidly after ethanol removal. In addition, the protein kinase A inhibitor Rp-cAMPs and protein phosphatase inhibitor okadaic acid both increased ethanol potentiation of thapsigargin while Sp-cAMPs, an activator of protein kinase A, decreased ethanol potentiation. Taken together, our findings suggest two mechanisms by which ethanol regulates grp78 transcription, both differing from the action of classical GRP inducers such as thapsigargin. One mechanism (potentiation) involves a protein phosphorylation cascade and potentiates the action of classical GRP inducers. In contrast, GRP78 induction by ethanol involves promoter sequences and a mechanistic pathway separate from that of the ethanol potentiation response or classical GRP78 inducers. These studies show that ethanol produces a novel and complex regulation of grp78 transcription which could be of particular importance during neuronal exposure to GRP-inducing stressors as might occur with central nervous system injury.

The “glucose-regulated proteins” (GRPs),1 GRP78 and GRP94, are among a unique set of ethanol-responsive genes induced in neural cell cultures by chronic ethanol exposure (1). The GRPs are a subgroup of molecular chaperones that participate in trafficking of glycoproteins. GRP78, the most well studied GRP, is a luminal endoplasmic reticulum (ER) protein that transiently associates with ER proteins undergoing glycosylation. “Classical” agents which increase expression of GRP78 and other GRP proteins include treatments that deplete ER calcium (A23187, thapsigargin), inhibit protein glycosylation (tunicamycin), or block vesicular trafficking (brefeldin A) (2). Conversely, alterations in GRP78 expression produce selective changes in protein trafficking (3).

Ethanol could increase GRP78 and GRP94 expression by altering ER calcium or glycoprotein trafficking through actions similar to classical inducers of GRP expression. However, several findings suggest ethanol induces GRP78 and GRP94 through a unique mechanism. First of all, ethanol increases GRP94 mRNA abundance to the same degree as GRP78, while the latter is more responsive to classical GRP inducing agents in our studies (1). Secondly, we found that ethanol concentration-response curves for GRP94, GRP78, and other ethanol-responsive genes are highly similar (1, 4–6), suggesting that ethanol could regulate ethanol-responsive genes through a common mechanism. In contrast, classical agents (tunicamycin, thapsigargin) that induce GRP78 and GRP94 do not regulate other ethanol-responsive genes (1).

Determining how ethanol regulates GRP expression could identify mechanism(s) involved in regulation of other ethanol-responsive genes. In this study, we examined the relationship between ethanol and other agents regulating GRP expression to define the site of action for ethanol. We focused on grp78 since regulation of this gene has been studied in great detail. For example, studies have identified that the same redundant cis-acting elements in the grp78 promoter confer responsive-

1 The abbreviations used are: GRP, glucose-responsive protein; ETGRs, ethanol-responsive genes; ER, endoplasmic reticulum; CAT, chloramphenicol acetyltransferase; cAMPs, adenosine cyclic 3′,5′-phosphorothioate; BFA, brefeldin A.

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EXPERIMENTAL PROCEDURES

Materials—Radioisotopes were purchased from DuPont NEN. All enzymes and reagents for recombinant DNA procedures were from Boehringer Mannheim. Brefeldin A was obtained from Epicentre Technologies. Other chemicals were of reagent grade from Sigma. Plasmids for Northern blot hybridization of GRP78 and all plasmids used for transient transfection analyses of grp78 promoter activity were from Dr. Amy Lee (University of Southern California). Plasmid p10, used for the majority of stable transfection studies, contains 1.25 kilobases of the rat grp78 promoter coupled to a chloramphenicol acetyltransferase (CAT) reporter gene (13). Other grp78 promoter constructs are described in Wooden et al. (14) and the legend to Fig. 4 or Fig. 5. Plasmid pM5VCA T contains the long-terminal repeat region from the Moloney murine sarcoma virus coupled to a CAT reporter gene and was used as a control for transient transfections.

Cell Culture and Transfection Analysis—NG108-15 cells were grown as described previously in Ham’s F-10 containing 10% NuSerum (Collaborative Research) or in defined culture medium (15). Cells were routinely subcultured at 7-10 day intervals and treated with ethanol or other drugs as described in the text. Control cultures were mock-treated and handled in an identical fashion to drug-treated cells.

Transient transfection of NG108-15 cells was done using Lipo- fectamine exactly as described by the supplier (Life Technologies, Inc.). Twenty-four hours after transfection, cells were treated with ethanol or other drugs as described under “Results.” CAT activity was assayed in cell lysates prepared by freeze-thawing in 0.25 m Tris, pH 8.0. CAT assays were performed as described by Seed and Sheen (16).

Stably transfected NG108-15 cells were isolated by co-transfection of cells with pSV neo (17) and a grp78 promoter-CAT construct (p10 or as indicated in text). The pSV neo plasmid contains the SV40 early promoter controlling expression for aminoglycoside 3’-phosphotransferase (I). Cells were transfected, selected for resistance to G418 (Life Technologies, Inc.) and clonal isolates were screened for CAT activity as described previously (15). Independent clones expressing CAT activity were screened for responsiveness to ethanol and GRP inducing agents.

Northern Blot Analysis—Total RNA from NG108-15 cells was analyzed by Northern hybridization as described previously (15). Hybridization probes were labeled using T7 polymerase (Stratagene). Template for the GRP78 hybridization probe was a hamster GRP78 cDNA clone isolated by Lee et al. (18). Hybridization with a probe for 18 S ribosomal RNA was used to detect variations in sample loading. Control hybridizations were also done using a probe for glyceraldehyde-3-phosphate dehydrogenase (Clontech). Following hybridization and autoradiography, membranes were stripped of probes by boiling in 0.1 × SSC, 0.01% SDS for 15 min and then rehybridized to allow probing of multiple membranes for multiple gene products. Autoradiograms were quantitated by computerized densitometry using a Microscan 1000 gel analyzer (Technology Resources Inc.). Multiple exposures were done to ensure linearity of the film response.

RESULTS

Ethanol Increases the Action of Classical Inducers of GRP78 mRNA—Using subtractive hybridization cloning, we recently showed that GRP78 and GRP94 are members of a group of ethanol-responsive genes in NG108-15 neuroblastoma × glioma cells (1). Our previous studies suggest ethanol may act differently than other agents that increase expression of GRP78 and GRP94. To further localize possible site(s) of ethanol action, we studied how ethanol in combination with classical GRP-inducing agents alters GRP78 mRNA abundance. For these studies, we used A23187, thapsigargin (Thap), or brefeldin A (BFA) to induce GRP78 so as to assess ethanol responses on agents with differing sites of action. The calcium ionophore A23187 increases intracellular Ca2+ by altering calcium permeability of cellular membranes while thapsigargin causes a selective depletion of endoplasmic reticulum (ER) calcium stores by inhibiting an ER calcium ATPase (19). The fungal metabolite, brefeldin A (BFA), blocks vesicular trafficking from the ER to the Golgi apparatus (20). All three of these agents produce large increases in GRP78 expression through increasing grp78 gene transcription (21).

Following treatment of NG108-15 cells with A23187, brefeldin A, or thapsigargin for 24 h in the presence or absence of 100 mM ethanol, steady state levels of GRP78 mRNA were measured by Northern slot blot analysis. Ethanol increased the induction of GRP78 mRNA by BFA, despite the latter agent producing a much larger increase in GRP78 mRNA abundance than seen with ethanol alone (Fig. 1, top). In contrast, only slight decreases in glyceraldehyde-3-phosphate dehydrogenase mRNA were seen with ethanol, BFA, or BFA + EtOH. Quan-
titative analysis showed that A23187, thapsigargin, and BFA increased GRP78 mRNA levels by 1.6-, 6.4-, and 8-fold, respectively (Fig. 1, bottom). In these experiments, ethanol alone increased GRP78 mRNA abundance by 58% (data not shown), a value similar to that seen in our previous studies (1). However, when used in combination, ethanol produced a 2-fold increase in the magnitude of GRP78 mRNA induction resulting from each of these other agents. This occurred despite thapsigargin or BFA producing much larger increases in GRP78 mRNA than seen with ethanol alone. Similarly, greater-than-additive increases in GRP78 protein were seen on Western blot analysis of cells treated with ethanol + thapsigargin compared to cells treated with either agent alone (data not shown).

Ethanol Increases Basal grp78 Transcription and Potentiates Thapsigargin-induced grp78 Promoter Activity in Stably Transfected NG108-15 Cells—The results of Fig. 1 indicate that ethanol induces GRP78 expression and increases the action of other GRP inducers. We will refer to these as "induction" and "potentiation" responses, respectively. Changes in GRP78 mRNA abundance seen with ethanol treatment could be due to increased grp78 gene transcription or altered GRP78 mRNA processing. To study the mechanism(s) underlying these actions of ethanol, we generated clonal isolates of NG108-15 cells stably transfected with a plasmid construct containing 1.25 kilobases of the hamster grp78 promoter coupled to a chloramphenicol acetyltransferase (CAT) reporter gene (13). Ethanol produced concentration-dependent increases in grp78 promoter activity in these stably transfected NG108-15 cells (Fig. 2A). Treatment with 100 mM ethanol for 24 h increased grp78 promoter activity to 167 ± 10% of control levels (mean ± S.E., p < 0.0001 versus control, single group t test, n = 13). Similar responses were observed with five different clonal isolates (not shown).

Treatment with 100 nM thapsigargin in the presence or absence of varying concentrations of ethanol produced a potentiation response with grp78 promoter activity (Fig. 2B), as seen with GRP78 mRNA abundance in Fig. 1. Thus, both the ethanol induction and potentiation responses occurred at the transcriptional level. The ethanol concentration-response for potentiation was similar to that of the induction response (Fig. 2A).

We calculated the degree of potentiation produced by ethanol using a formula to correct for increases in grp78 promoter activity due to simple additivity (see legend to Fig. 1). This formula results in a value of 1.0 if ethanol produces only an additive response. Fig. 2C shows the potentiation response as a function of ethanol concentration. The average fold potentiation by 100 mM ethanol seen with 100 nM thapsigargin was 2.4 ± 0.3 (p < 0.0001 versus simple additivity, single group t test, n = 8).

Ethanol Acts Uniquely in Producing a Potentiation of grp78 Promoter Activity—If the ethanol potentiation response occurs by ethanol acting at a site distinct from classical GRP inducers, then ethanol should potentiate even at maximally effective concentrations of thapsigargin. Therefore, we studied the thapsigargin concentration-response for grp78 promoter activation in the presence or absence of ethanol. Ethanol (100 mM) produced similar potentiations at all thapsigargin concentrations studied (Fig. 3A), including maximally effective thapsigargin levels (>50 nM).

Consistent with a separate site of ethanol action, we found that ethanol also potentiated the action of other inducers of grp78 promoter activity. Fig. 3B shows that ethanol potentiated both thapsigargin and tunicamycin, an inhibitor of core glycosylation (Fig. 3B). Similar ethanol potentiation was also seen with BFA and A23187 (data not shown).

If ethanol produced a potentiation response simply by acting at a site parallel to other inducers of GRP expression, we expected that classical GRP inducers working at different sites should also potentiate one another. For example, BFA or tunicamycin should potentiate the action of thapsigargin or A23187. We found, however, that when tunicamycin was added to saturating levels of thapsigargin there was only an additive response (potentiation ratio = 1.17) compared to the potentiation produced by ethanol on thapsigargin or tunicamycin action (Fig. 3B). Moreover, ethanol was still able to produce a potentiation response when added together with both thapsigargin and tunicamycin (Fig. 3B). Similar findings resulted when various pairings of BFA, A23187, tunicamycin, or thapsigargin were used. None of these agents potentiated the action of one another (data not shown). These results further suggested that ethanol produces the potentiation response by a unique mechanism which interacts "downstream" with the signaling cascade triggered by classical GRP inducers.

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through Separate grp78 Promoter Elements—cis-Acting elements in the grp78 promoter which mediate response to classical GRP inducing agents such as thapsigargin have been characterized extensively (9, 14, 22). A bipartite element spanning the region −169 to −88 of the rat grp78 promoter is capable of a fully functional response to thapsigargin or other classical GRP inducing agents (8). Subdivisions of this element appear to exist with a “core” region containing constitutive DNA-binding protein activity while inducible protein binding occurs in the −135 to −169 region (Fig. 4A). In addition, Lee and co-workers have shown recently that a proximal “CCAAT” element present in the rat grp78 promoter (C1 in Fig. 4A) appears indispensable for the action of classical GRP inducing agents. Using a series of grp78 promoter deletion mutants, we investigated whether the induction and potentiation responses to ethanol occur through the same promoter motif used for thapsigargin induction.

Progressive deletions of the grp78 promoter showed that, as expected, thapsigargin responsiveness decreased with constructs proximal to −169 (Fig. 4C). The relative ethanol potentiation response persisted with deletion to −130, although this promoter construct had a reduced response to thapsigargin. The grp78 promoter response to thapsigargin or thapsigargin + ethanol virtually disappeared with deletion to the −104 position (5′(−104)CAT) but this construct had an intact ethanol induction response (Fig. 4B). Deletion further to the −85 position did eliminate ethanol induction (Fig. 4B). Basal activities for 5′(−85)CAT and 5′(−104)CAT were 41 ± 6 versus 29 ± 6 cpm/μg (mean ± S.D. from 3 independent experiments), respectively, suggesting that decreases in basal activity did not account for loss of ethanol responsiveness with 5′(−85)CAT. Stable transfection analysis of the 5′(−85)CAT construct showed similar results with multiple clonal isolates having no significant induction by ethanol although their basal activities varied widely, all well above background levels. Control transfections using a Moloney sarcoma virus long terminal repeat coupled to CAT (pMSVCAT) (15) showed only slight decreases in CAT activity with ethanol, thapsigargin, or EtOH + Thap (Fig. 4, B and C).

Ethanol Potentiation of Thapsigargin Requires grp78 Promoter Sequences Distinct from Those Mediating Ethanol or Thapsigargin Induction—The promoter deletion analysis described in Fig. 4 did not distinguish whether the ethanol potentiation response required the same sequences utilized by thapsigargin induction, ethanol induction, or both. We therefore performed a detailed analysis of the grp78 promoter using a series of linker-scanning mutants which have been characterized extensively (14).

Fig. 5 shows that the LS90 and LS120 linker-scanning mutants had large decreases in thapsigargin induction, similar to previous results by Lee and co-workers (8). Although LS120 and LS90 had reduced responses to thapsigargin, ethanol still potentiated thapsigargin nearly as well as with the 5′(−456)CAT parent construct (Fig. 5B). Conversely, the potentiation response was totally eliminated with the LS100 mutation although this construct showed a nearly 7-fold induction by thapsigargin. All of the linker-scanning mutations had similar inductions by ethanol alone (Fig. 5A). A double linker scanning mutant (LS130/90) which had no response to thapsigargin in NG108-15 cells also showed no potentiation by ethanol (data not shown). Stable transfection analyses using the LS100 construct confirmed the transient transfection data with multiple clonal isolates having a robust induction by thapsigargin but no potentiation response with ethanol + thapsigargin (Fig. 5C).

Ethanol Potentiation of Thapsigargin-induced grp78 Promoter Activity Occurs Rapidly—The promoter deletion and linker-scanning studies (Figs. 4 and 5) suggested that the ethanol induction and potentiation responses represented actions of ethanol at two separate sites of the grp78 promoter. To further study and contrast the mechanism(s) of the ethanol induction and potentiation responses, we performed time course and wash-out experiments using NG108-15 cells stably transfected with the pI10 grp78 promoter construct. If the ethanol responses involved a protein phosphorylation cascade as implicated for classical GRP inducers (11, 12), we expected a rapid onset and decay of ethanol action. Time course analysis

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showed that ethanol potentiation of grp promoter activity was maximal as soon as thapsigargin-induced changes in GRP78 expression could be detected (6 h in Fig. 6). In contrast, ethanol-induced changes in grp78 basal promoter activity first became detectable by 16–24 h after treatment.

To determine the relative duration of the biochemical event(s) underlying the ethanol potentiation response, NG108-15 cells stably transfected with pI10 were pretreated with ethanol for 24 h, followed by exposure to thapsigargin for 6 h in the presence or absence of ethanol. Shorter time intervals for ethanol withdrawal were not tested due to variation in the thapsigargin response at brief stimulation times. Cells pretreated with ethanol, rinsed, and then exposed to thapsigargin alone for 6 h showed a grp78 promoter response similar to cells without ethanol pretreatment (Fig. 6B). Ethanol pretreatment did not significantly alter the potentiation seen with a subsequent 6-h exposure to thapsigargin + ethanol (Fig. 6B). Taken in total, these data suggest that the potentiation response occurred rapidly and decays within 6 h if ethanol is removed. The ethanol potentiation response persisted as long as ethanol was present since cells pretreated with ethanol for up to 48 h, followed by 6 h of thapsigargin + ethanol, showed a potentiation response similar to cells exposed to only 6 h of ethanol (data not shown).

Inhibitors of Protein Kinase A and Protein Phosphatases Increase Ethanol Potentiation of GRP78—Recent reports have shown that the protein tyrosine kinase inhibitor genistein will inhibit GRP78 induction by thapsigargin while okadaic acid, a protein phosphatase inhibitor, slightly increases the GRP78 response to thapsigargin (11, 12). Furthermore, Cox et al. (10) have identified a transmembrane protein kinase required for GRP78 induction in yeast. Thus, a protein phosphorylation cascade may mediate the GRP78 response to thapsigargin and could also be a site for ethanol action in either the induction or potentiation responses. We therefore studied the action of protein kinase or phosphatase inhibitors on ethanol potentiation and induction of GRP78 expression. These experiments were done using 6-h treatment times to avoid cell toxicity seen on prolonged incubation with the various inhibitors. The 6-h treatment time allowed study of inhibitor action on GRP78 responses to thapsigargin or ethanol + thapsigargin but precluded definitive conclusions on GRP78 induction by ethanol alone since this latter response was of small magnitude at early time points (see Fig. 5).

Both okadaic acid and the protein kinase A inhibitor Rp-cAMPS caused large increases in the response to thapsigargin + ethanol while minimally changing grp78 promoter responses to either thapsigargin or ethanol alone (Fig. 7). In contrast, the protein kinase A activator, Sct-cAMPS, caused decreases in ethanol potentiation of thapsigargin action. Similar to other investigators, we found that genistein (100 μM) inhibited the induction of grp78 promoter activity by thapsigargin (data not shown). In the presence of genistein, the response to ethanol + thapsigargin was actually slightly less than that of thapsigargin alone. In all these studies, we did not observe any significant change in cell number, cellular protein content, or cell morphology with 6-h exposures to multiple drug combinations.

**DISCUSSION**

Our previous studies identified the molecular chaperones Hsc70, GRP78, and GRP94 among a unique set of ethanol-responsive proteins whose expression increases in neural cells following prolonged exposure to ethanol (1). The regulation of GRP78 and GRP94 has been studied extensively and shown to
involve a complex signal transduction cascade that relays information from the ER to the nucleus. This ER signaling pathway can be activated by diverse stimuli which alter protein processing and trafficking through the ER. Our results here show that ethanol can increase grp78 transcription in two distinct manners: a direct induction by ethanol and a unique synergistic interaction with the ER signaling cascade regulating GRP expression.

Depletion of ER calcium (thapsigargin, A23187), inhibition of protein glycosylation (tunicamycin), interference with vesicular trafficking (brefeldin A), or overproduction of malfolded glycoproteins have all been shown to increase expression of GRP78, GRP94, and several other ER proteins that function as molecular chaperones (2). Despite differing sites of action for these GRP inducing agents, they all are thought to trigger a common signaling cascade involving perhaps multiple protein kinases that lead to increased grp gene transcription. Indeed, diverse agents such as brefeldin A, thapsigargin, and A23187 have all been shown to require the same cis-acting elements in the grp78 promoter (7, 8).

Studies presented here showed that like classical GRP inducers, ethanol increased GRP78 expression at the level of
gene transcription. However, the addition of ethanol to a variety of classical GRP inducers produced an absolute response that was much greater than additive even at maximally effective concentrations of classical inducers such as thapsigargin (Figs. 1–3). This potentiation of classical GRP inducing agents produced ethanol-responsive increases in GRP78 expression that were of much greater absolute magnitude than seen with induction by ethanol alone. The potentiation by ethanol suggested a synergistic interaction between two separate mechanisms. This hypothesis was supported by finding that ethanol potentiated multiple classical GRP78 inducing agents having different sites of action.

Since ethanol produces increases in both GRP94 and GRP78 mRNA (1), we initially questioned whether ethanol might also act through the same signaling cascade as classical GRP inducers. Together with our previous results (1), however, studies here have shown in a number of ways that ethanol acts in a manner distinct from classical GRP inducers. As mentioned above, ethanol potentiation of classical GRP inducers inferred the existence of an ethanol-responsive signaling cascade separate from that triggered by classical GRP inducers. Secondly, ethanol induced grp78 transcription or potentiated thapsigargin-induced grp78 transcription through promoter element(s) which differ from those defined as mediating the action of classical GRP inducing agents. Finally, ethanol potentiation of GRP78 expression also differs from classical GRP inducers in response to inhibitors of protein phosphorylation/dephosphorylation.

Perhaps the most straightforward explanation for our findings would be that ethanol and thapsigargin increase grp78 transcription through separate pathways (induction responses) that can interact (potentiation responses) at some point “distal” to the ER events targeted by classical GRP inducers. However, several of our results suggested that the ethanol induction and potentiation responses actually represent separate actions of ethanol that both differ from mechanisms of classical GRP inducers. First, the rapid time course of the potentiation response contrasted with the more gradual induction of grp78 transcription by ethanol alone. Secondly, the potentiation response was eliminated by linker-scanning mutation of sequences at −109 to −100 in the grp78 promoter (LS100 in Fig. 5) while ethanol induction and thapsigargin induction remained intact in this construct. Finally, the potentiation response was markedly increased by R<sub>i</sub>-cAMPS or okadaic acid while these agents produce minimal to no change in grp78 promoter responses to ethanol or thapsigargin alone. Thus, the potentiation response appeared to involve a phosphorylation cascade that interacted with the thapsigargin pathway while ethanol induction may involve more slowly evolving biochemical events such as changes in transcription factor abundance. The rapid reversal of the potentiation response following ethanol wash-out was consistent with a short-lived regulatory event such as phosphorylation. Whether a common proximal action of ethanol underlies both the induction and potentiation responses remains to be determined.

The potentiation response required promoter sequences at −109 to −100 of the grp78 promoter. This region has been shown to contain constitutive protein binding sites (9) and is directly adjacent to a CCAAT site (C1 in Fig. 4A). The C1 site has recently been shown to be crucial for the action of classical GRP inducers (22). The CBF (CCAAT-binding factor) protein that occupies this site may respond directly to alterations in cellular calcium or interact with inducible binding factors at the core region of the grp78 promoter (22). Ethanol could thus potentiate thapsigargin by acting on a protein factor binding adjacent to C1. This potentiation factor might alter the interaction of core protein(s) with CBF.

Several recent studies have documented a role for both serine/threonine (10) and tyrosine-protein kinases (12) in the induction of GRP78 by classical agents such as thapsigargin. Our studies here suggested that the ethanol potentiation response involved a protein phosphorylation cascade different from that mediating action of thapsigargin. Since a phosphatase inhibitor (okadaic acid) and a protein kinase A inhibitor (R<sub>i</sub>-cAMPS) produced similar effects on ethanol potentiation, this suggested that a linked phosphorylation/dephosphorylation cascade was involved in the potentiation response. For example, a phosphatase-induced de-phosphorylation event inhibitory to thapsigargin action could, in turn, be inhibited by CAMP-dependent protein kinase-dependent phosphorylation. Ethanol could act directly on this CAMP-dependent protein kinase-phosphatase path or perhaps through another signaling cascade converging on the same protein(s) acted upon by the phosphatase-CAMP-dependent protein kinase system. Inhibition of ethanol potentiation by a CAMP-dependent protein kinase activator (S<sub>p</sub>-cAMPS) further supports the role of CAMP-dependent protein kinase-dependent phosphorylation in the mechanism of the potentiation response. Although other explanations could be offered to explain our results and await further studies for clarification of the exact signaling cascade mediating the potentiation response, our findings here represent a first step in understanding the mechanism of ethanol-regulated gene transcription. The possible role of CAMP-dependent protein kinase is particularly intriguing since there is a large amount of experimental literature documenting changes in cyclic AMP signal transduction with acute and chronic exposure to ethanol (see Ref. 23 for review).

In contrast to the ethanol potentiation and thapsigargin induction responses, ethanol induction of GRP78 transcription was not significantly altered in any of the linker scanning mutants tested. However, ethanol induction was lost when grp78 promoter sequences distal to −85 were deleted. Since the −104 deletion mutant still retained ethanol induction, the 20-base pair region between −85 and −104 should contain sequences crucial to the ethanol induction response. Since single linker scanning mutants in this region (LS80, LS90, and
LS100) did not alter ethanol induction, this suggested either that the response element may be diffusely represented across this area or that redundant ethanol induction sequences are contained in other regions of the linker scanner mutants.

For example, the -5′(-85)CAT construct does not contain any intact copy of the repeated CCAAT motifs. If the ethanol induction response occurred through a CCAAT motif, then we might expect that no single linker scanner mutation would eliminate the ethanol induction due to the redundancy of these elements in the grp78 promoter. An alternate explanation for our results would be that the -85 dilution mutant simply had lost sufficient basal activity to register an ethanol induction response rather than representing deletion of specific ethanol-responsive cis-acting sequences. We do not believe this to be the case since -5′(-85)CAT had identical basal activity to the -5′(-104)CAT construct which still retained ethanol responsiveness. Furthermore, multiple clonal isolates stably transfected with -5′(-85)CAT were also unresponsive to ethanol even though basal activity varied in these isolates by more than 2 orders of magnitude. Assaying artificial promoter constructs containing the -85 to -104 region should confirm whether an ethanol induction response element resides in this area.

Regulation of GRP78 by ethanol could have important functional implications for the central nervous system, particularly in settings of neuronal injury. GRP78 is among several stress proteins that are induced in neural or glial cells following ischemic injury (24). Although the exact physiological role of such protein inductions following central nervous system injury remains to be determined, several reports have suggested a neuroprotective effect of stress protein inductions (25, 26). Based upon our results, ethanol would be expected to potentiate the induction of GRP78 by ischemia or other forms of central nervous system injury.

As we suggested previously, induction of GRP78 expression by chronic ethanol exposure might also alter protein trafficking, producing changes in the abundance of membrane or secretory proteins. Dorner et al. (3) have indeed shown that increased GRP78 expression can cause a selective inhibition of protein secretion. Changes in GRP78 expression consequent to chronic ethanol exposure could thus have functional consequences for the central nervous system if alterations occur in the membrane abundance of neurotransmitter receptors known to be targets of acute ethanol action (27).

There are a growing number of reports documenting ethanol-responsive changes in gene expression. The combination of these molecular events may contribute to alterations in the nervous system seen with chronic ethanol exposure. Further study on how ethanol regulates GRP78 expression may have important implications for mechanisms underlying regulation of other ethanol-responsive genes.

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REFERENCES

1. Miles, M. F., Wilke, N., Elliot, M., Tanner, W., and Shah, S. (1994) Mol. Pharmacol. 46, 873–879
2. Lee, A. S. (1992) Curr. Opin. Cell Biol. 4, 267–273
3. Dorner, A. J., Wasley, L. C., and Kaufman, R. J. (1992) EMBO J. 11, 1563–1571
4. Miles, M. F., Diaz, J. E., and DeGuzman, V. (1992) Biochim. Biophys. Acta 1138, 265–272
5. Miles, M. F., Barhite, S., Sganga, M., and Elliott, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10831–10835
6. Gayer, G. G., Gordon, A., and Miles, M. F. (1991) J. Biol. Chem. 266, 22275–22284
7. Liu, E. S., Ou, J., and Lee, A. S. (1992) J. Biol. Chem. 267, 7128–7133
8. B. L. W. W., Alexandre, S., Cao, X., and Lee, A. S. (1993) J. Biol. Chem. 268, 12003–12009
9. Li, W. W., Sistenon, L., Moriimoto, R. I., and Lee, A. S. (1994) Mol. Cell. Biol. 14, 5533–5546
10. Cox, J. S., Shamu, C. E., and Waller, P. (1993) Cell 73, 1197–1206
11. Cao, X., Zhao, Y., and Lee, A. S. (1995) J. Biol. Chem. 270, 494–502
12. Price, B. D., Mannheim-Rodman, L. A., and Calderwood, S. K. (1992) J. Biol. Chem. 267, 549–552
13. Resendez, E., Altonello, J. W., Grafaky, A., Chang, C. S., and Lee, A. S. (1985) Mol. Cell. Biol. 5, 1212–1219
14. Wooden, S. K., Li, L.-J., Navarro, D., Qadri, I., Pereira, L., and Lee, A. S. (1991) Mol. Cell. Biol. 11, 5612–5623
15. Miles, M. F., Diaz, J. E., and DeGuzman, V. S. (1991) J. Biol. Chem. 266, 2409–2414
16. Seed, B., and Sheen, J.-Y. (1988) Genes Dev. 2, 721–727
17. Southern, P. J., and Berg, P. (1982) J. Mol. Appl. Genet. 1, 327–341
18. Lee, A. S., Delegante, A. M., Baker, V., and Chow, P. C. (1983) J. Biol. Chem. 258, 597–603
19. Thaistrum, G., Cullen, P. J., Drobatz, B. K., Hanley, M. R., and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470
20. Doms, R. W., Russ, G., and Yewdell, J. W. (1989) J. Cell Biol. 106, 61–72
21. Lee, K.-M., Toscas, K., and Villereal, M. L. (1991) J. Biol. Chem. 268, 9945–9948
22. Roy, B., and Lee, A. S. (1995) Mol. Cell. Biol. 15, 2263–2274
23. Gordon, A. S., Mochly-Rosen, D., and Diamond, I. (1992) in G-proteins Signal Transduction and Disease (Milligan, G., and Wakelin, M., eds) pp. 191–216, Academic Press, London
24. Lowenstein, D. H., Gwinn, R. P., Seren, M. S., Simon, R. P., and M. D., T. K. (1994) Mol. Brain Res. 22, 299–308
25. Lowenstein, D. H., Chan, P. H., and Miles, M. F. (1991) Neuron 7, 1053–1060
26. Barbe, M. F., Tytell, M., Gower, D. J., and Welch, W. J. (1988) Science 241, 1817–1820
27. Buc, K. J., and Harris, R. A. (1991) Alcohol. Clin. Exp. Res. 15, 460–470