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Promitogenic Effects of Ethanol, Methanol, and Ethanolamine in Insulin-Treated Fibroblasts

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ABSTRACT. The zinc-dependent potentiating effect of ethanol (EtOH) on insulin-stimulated DNA synthesis was studied with a focus on the possible site of EtOH action and the ability of other alcohols to elicit similar promitogenic effects. In serum-starved (27 hr) NIH 3T3 fibroblasts, 200–300 mM methanol (MeOH) and 0.1–1.5 mM ethanolamine (Etn), but not 3- to 9-carbon normal alcohols, enhanced the effect of insulin on DNA synthesis to varying extents. The promitogenic effects of EtOH and MeOH, but not that of Etn, required the presence of 15–25 μM zinc. The potentiating effects of Etn were enhanced by 5 mM choline (Cho) and inhibited by 1–3 mM hemicholinium-3 (HC-3), an inhibitor of Cho transporter and Cho kinase. In the presence of 15 μM zinc, 40 mM EtOH, which had no effect on its own, inhibited the potentiating effects of Cho and enhanced the inhibitory effects of HC-3 on synergistic stimulation of DNA synthesis by Etn and insulin. On the other hand, both Cho and HC-3 partially inhibited the promitogenic effect of 80 mM EtOH in the presence of 25 μM zinc. After a 10-min incubation, EtOH decreased the amount of cell-associated [^{14}C]Cho in the absence but not in the presence of HC-3. After a 40-min incubation, Cho (5 mM) partially inhibited the cellular uptake as well as the metabolism of [^{14}C]Etn. Whereas after the 40-min incubation 80 mM EtOH had no effects on Etn metabolism, in the absence of Cho it decreased the amount of cell-associated [^{14}C]Etn. However, EtOH had no detectable effects on cell association of [^{14}C]Etn after the 10-min incubation. The results suggest that in NIH 3T3 fibroblasts EtOH is a remarkably specific promitogen, and that it may act via a cell membrane site(s), also regulated by Cho (agonist) and HC-3 (antagonist), which can influence membrane binding and the promitogenic activity of Etn. BIOCHEM PHARMACOL **60**;9:1391–1398, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. ethanol; methanol; ethanolamine; choline; insulin; DNA synthesis

In many cellular systems, EtOH§ has been found to inhibit the effects of growth factors, including insulin and insulin-like growth factor, on cell growth [1–7]. The growth-inhibitory effects of EtOH are often accompanied by increased apoptotic cell death [8–12]. It is reasonable to assume that these negative effects of EtOH on cell growth provide the cellular basis for alcohol abuse-related growth abnormalities in the growing embryo and fetus, which can lead to fetal alcohol syndrome [13, 14].

In contrast to the above observations, in certain cell lines and under specific conditions EtOH may actually enhance mitogenesis. Thus, at a low (0.1%) concentration, EtOH has been found to stimulate preimplantation development of murine embryos, resulting in an improved implantation rate [15, 16]. EtOH also stimulates proliferation of cells in the neocortical subventricular zone in rats [17] and that of astrocytes *in vitro* [18]. Furthermore, alcohol intake results in hyperproliferation of gastrointestinal mucosa [19, 20].

The potential stimulatory effects of EtOH on mitogenesis drew our attention because epidemiological studies have indicated that alcohol abuse can be a risk factor for certain human cancers [21–24], and because chronic exposure to EtOH has been shown to increase proliferation of various types of cancer cells *in vitro* [25–27]. Although the possibility has been raised that EtOH acts in part as a solvent for carcinogens or as an inducer of microsomal enzymes that activate chemical carcinogens [21], it is also reasonable to assume that EtOH promotes carcinogenesis because under specific conditions it stimulates DNA synthesis and cell proliferation. In turn, by increasing DNA synthesis, EtOH could stabilize carcinogen/mutagen-induced structural changes in DNA, thereby increasing the chances of cell transformation.

According to our interest, during the last few years the focus of our investigation has been to develop a suitable cellular model system where the stimulatory effect of EtOH on DNA synthesis and possibly cell transformation can be studied easily. Thus far, this work has revealed that in mouse NIH 3T3 fibroblasts EtOH can greatly enhance the stimulatory effects of insulin, insulin-like growth factor-I,

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§ *Abbreviations*: EtOH, ethanol; MeOH, methanol; Etn, ethanolamine; PEtn, phosphoethanolamine; PtdEtn, phosphatidylethanolamine; Cho, choline; PCho, phosphocholine; PtdCho, phosphatidylcholine; HC-3, hemicholinium-3; MAP, mitogen-activated protein; and DMEM, Dulbecco's modified Eagle's medium.

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several other peptide growth factors, and sphingosine 1-phosphate on DNA synthesis by a strictly zinc- and calcium-dependent mechanism [28–32]. We have also found that in many human skin fibroblast lines EtOH can enhance the effect of calcium on cell proliferation [33].

Whereas the above studies have clarified that zinc is required to prevent the inhibitory effect of EtOH on insulin-induced activation of p42/p44 MAP kinases [31], and that EtOH may act in part by delaying zinc plus insulin-induced premature expression of cyclin E [32], many questions relating to the mechanism of EtOH action have remained to be solved by future studies. Our goal in the present work was to determine whether other alcohols might be able to reproduce, at least in part, the promitogenic effect of EtOH; we hope that this information will help to determine the exact cellular site of EtOH action. We found that only MeOH and Etn, but not 3- to 9-carbon normal alcohols, can fully or partially mimic the potentiating effect of EtOH on insulin-stimulated DNA synthesis, indicating a high degree of specificity of EtOH action. Furthermore, our data suggest a complex interaction between the sites, presumably located at the cell membrane, which mediates the effects of EtOH and Etn on DNA synthesis.

MATERIALS AND METHODS

Materials

Spectroscopic grade EtOH and MeOH were bought from Aldrich/Sigma and were redistilled prior to use; the first ~35% fraction of each distillate was discarded to eliminate possible traces of benzene. Insulin was purchased from Boehringer Mannheim; HC-3, zinc chloride, Cho chloride, and calcium chloride were bought from the Sigma Chemical Co. [2-^{14}C]Etn (50 mCi/mmol) and [*methyl*-^{14}C]Cho (50 mCi/mmol) were purchased from Amersham, and [*methyl*-^{3}H]thymidine (500 mCi/mmol) was obtained from Dupont NEN. Tissue culture reagents, including fetal bovine serum and DMEM, were bought from GIBCO-BRL.

Cell Culture and Preparation of Stock Solutions for Zinc

Mouse embryo NIH 3T3 fibroblasts (American Type Culture Collection) were cultured in 10% fetal bovine serum-containing DMEM as indicated earlier [34]; DMEM contains about 28 μM Cho and 1.8 mM calcium but no zinc. For these series of experiments, the fibroblasts were cultured continuously for up to 40 passages. Since at higher concentrations zinc precipitates (in the form of insoluble salts) in DMEM, stock solutions of zinc were made up in water, and 10-μL aliquots (filtered) were delivered to the cells.

Labeling of Cellular DNA with [^{3}H]Thymidine

Fibroblasts were grown in 12-well tissue culture dishes to ~40% confluency in the presence of 10% serum; then they were washed, and incubated in serum-free DMEM for 24 hr. The cells were washed again 3 hr prior to the treatments. When applicable, the cells (70–80% confluent) were treated first (in serum-free medium; incubation volume, 0.75 mL) with 1–3 mM HC-3 for 30 min, then with 25 μM zinc chloride for 10 min, then with EtOH, MeOH, or Etn (Cho) for 10 min, and finally with insulin (in the presence of zinc and alcohols) for 18 hr; [*methyl*-^{3}H]thymidine (1 μCi/well) was present during the last 60 min of the treatments. The cells were washed twice with PBS, then four times with 5% trichloroacetic acid, and finally twice with absolute EtOH. The acid-insoluble material was redissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated ^{3}H activity in a liquid scintillation counter. Cell numbers in ~80% confluent cultures were ~1.5 to 1.7 × 10^{5}/well.

Measurement of Cell-Bound ^{14}C-Labeled Etn, Cho, and Its Metabolites

Fibroblasts were grown in 12-well plates to confluency in the presence of 10% serum. Then the cells were serum-starved for 24 hr, washed again, and incubated in serum-free medium for 10–40 min in the presence of 50 μM [^{14}C]Etn (855,000 dpm/well in Fig. 5, and 1,613,000 dpm/well in Fig. 6) or [^{14}C]Cho (936,000 dpm/well) and other additions as indicated in the text. At the conclusion of the incubations, the incubation medium was aspirated, and the cells were washed rapidly (within 30 sec) with 2 × 4 mL serum-free medium followed by the addition of ice-cold MeOH (1.5 mL) to the wells; finally, the MeOH extracts were transferred to tubes containing chloroform. After phase separation, [^{14}C]Etn/[^{14}C]Cho and [^{14}C]PEtn/[^{14}C]PCho were separated on a Dowex-50W (H^{+}) column, and [^{14}C]PtdEtn and [^{14}C]PtdCho were separated by one-dimensional TLC as described elsewhere [35].

Statistical Analysis

Data derived from 3 independent experiments and from 3–6 independent samples in the same experiment are expressed as means ± SEM and means ± SD, respectively. The unpaired *t*-test was used to evaluate statistical significance; P values of < 0.05 to 0.01 were considered significant.

RESULTS

Comparison of the Effects of 1- to 9-Carbon Normal Alcohols on DNA Synthesis

In the presence of 25 μM zinc, 60–100 mM concentrations of EtOH greatly enhanced the relatively small (about 8-fold) stimulatory effect of insulin on DNA synthesis (Fig. 1). We should note here that in previous experiments [31] we made up the zinc stock solution in DMEM, in which a smaller fraction of zinc was precipitated. This accounts for the discrepancy that whereas previously 40 μM zinc was

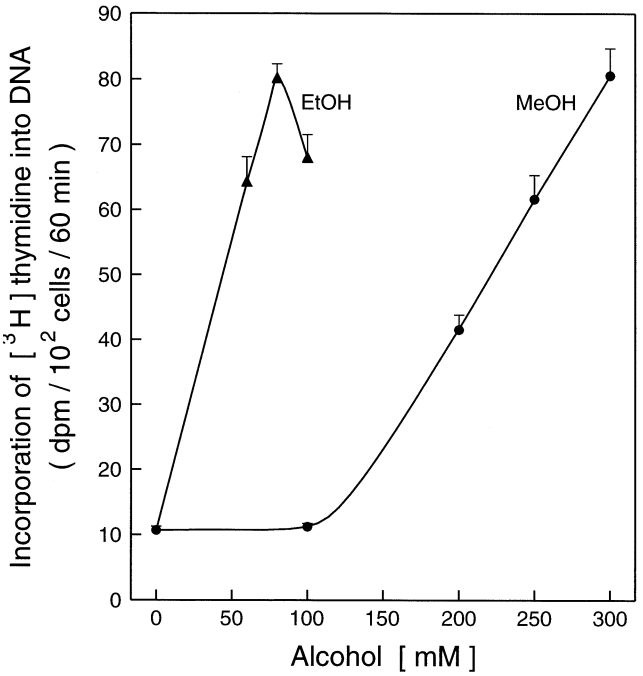

FIG. 1. Zinc-dependent potentiating effects of EtOH and MeOH on insulin-stimulated DNA synthesis. Serum-starved (27 hr) NIH 3T3 fibroblasts first were treated with 25 μM zinc for 20 min, followed by treatments with 500 nM insulin in the presence of 60–100 mM EtOH (▲) or 100–300 mM MeOH (●). Each point represents the mean ± SD of six separate samples in a single experiment using the same passage of cells. Similar results were obtained in two other experiments, each performed in triplicate.

required for maximum potentiation of the EtOH effect [31], presently, when the zinc stock solution was made up in water to avoid precipitation, 25 μM zinc was maximally effective. In the presence of zinc, but not in its absence, 200–300 mM MeOH also potentiated the effect of insulin on DNA synthesis (Fig. 1). An increase in MeOH concentration above 300 mM resulted in less potentiating effects (not shown). Since the differences in the concentration-dependent effects of EtOH and MeOH appeared to reflect the approximately 3-fold difference in their lipid solubility [36], we also examined the effects of 3- to 9-carbon normal alcohols to see if an apparent relationship between lipid solubility and stimulation of DNA synthesis was maintained. However, none of these alcohols had any stimulatory effects on DNA synthesis when used in the concentration range of 0.1 to 60 mM in both the absence and presence of 25 μM zinc and/or 500 nM insulin (not shown).

As we reported earlier [31], EtOH had easily detectable promitogenic effects only if it was added to the cells only once without sealing the wells. Under this condition, the concentration of EtOH decreases by approximately 50% during a 4-hr incubation period [15]. In the present study, we also found that MeOH had much smaller promoting effects on insulin-dependent DNA synthesis if the wells were sealed during the treatment period.

Combined Effects of EtOH, Etn, Cho, and HC-3 on DNA Synthesis

We have shown previously that in serum-starved NIH 3T3 cells Etn also can enhance, although more modestly than EtOH, the effect of insulin on DNA synthesis [37]. Furthermore, the promitogenic effect of Etn is enhanced by 5 mM Cho [37]. Because Cho also inhibits Etn uptake, it appeared that Etn most probably acted via a cell surface membrane site. Because the promitogenic effects of EtOH and Etn/Cho appeared to be highly specific, we considered that EtOH and Etn/Cho might act on DNA synthesis via a similar membrane site(s). For these reasons, we first examined how EtOH might affect DNA synthesis induced by Etn plus insulin.

In preliminary experiments, we determined that Etn had about the same effects on insulin-stimulated DNA synthesis in the absence or presence of 15 μM zinc, whereas 25 μM zinc decreased its effects by 20–25%. Although EtOH has optimal effects in the presence of 25 μM zinc, we wanted to optimize the effect of Etn so that at the same time EtOH still remained an effective promitogen. Therefore, in the following experiments, we examined the promitogenic effects of Etn and EtOH in the presence of 15 μM zinc. Etn, when used at 0.25 mM, approximately doubled the stimulatory effect of insulin; an increase in Etn concentration to 1.5 mM only slightly increased its effect (Fig. 2). The addition of 40 mM EtOH had no effects on insulin-stimulated DNA synthesis in either the absence or presence

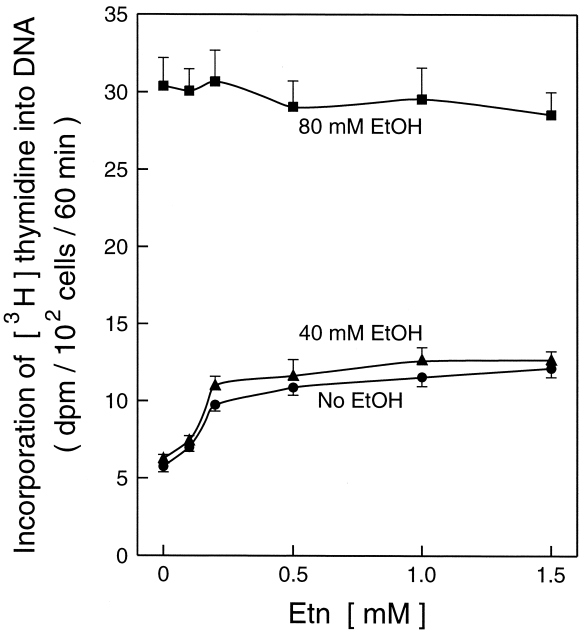

FIG. 2. Effects of EtOH and Etn on insulin-stimulated DNA synthesis. Serum-starved (27 hr) NIH 3T3 fibroblasts first were treated with 15 μM zinc for 20 min, followed by treatments with 500 nM insulin for 18 hr in the presence of 0.1 to 1.5 mM Etn (●), Etn plus 40 mM EtOH (▲), or Etn plus 80 mM EtOH (■). Each point represents the mean ± SD of three separate samples in a single experiment representative of three similar experiments.

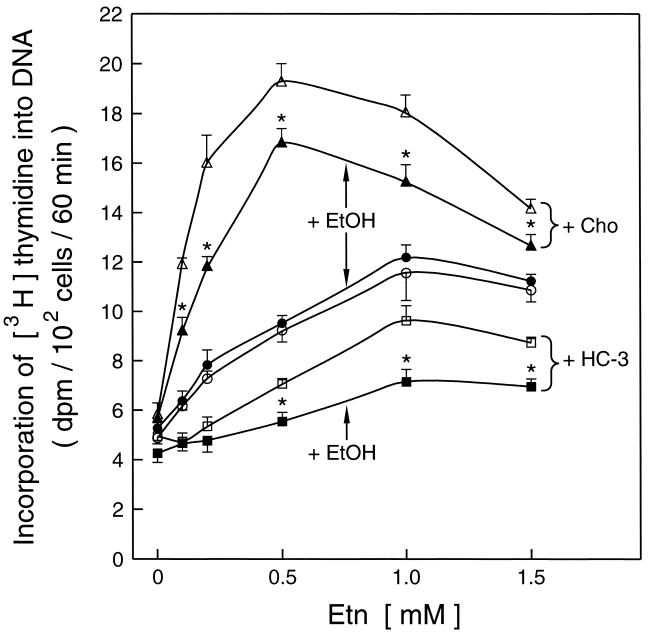

FIG. 3. Effects of EtOH, Cho, and HC-3 on DNA synthesis stimulated by insulin plus Etn. Serum-starved NIH 3T3 fibroblasts were first treated with 15 μM zinc for 20 min, followed by treatments with 500 nM insulin for 18 hr in the presence of 0.1 to 1.5 mM Etn (○-●), Etn plus 5 mM Cho (△-▲), or Etn plus 1 mM HC-3 (□-■) in the absence (open symbols) or presence (closed symbols) of 40 mM EtOH. Each point represents the mean ± SEM of three independent experiments, each performed in triplicate. Key: (*) significantly ($P < 0.01$ to 0.05) smaller than the corresponding value in the absence of EtOH (unpaired *t*-test).

of Etn (Fig. 2). Furthermore, under this condition, Etn did not affect the larger combined effects of 80 mM EtOH and insulin (Fig. 2). However, it should be noted that when this experiment was repeated in the presence of 25 μM zinc, 0.5 to 1.5 mM Etn slightly (10–16%) decreased the larger combined effects of insulin and 80 mM EtOH (data not shown). While these data do not suggest strong competition between the effects of EtOH and Etn, they clearly indicate that their effects are non-additive.

As mentioned earlier, Cho paradoxically increases the promitogenic effects of Etn. Interestingly, 40 mM EtOH was found to decrease significantly the potentiating effects of 5 mM Cho on DNA synthesis stimulated by Etn plus insulin, with the greatest inhibitory effects recorded at lower (0.1 to 0.25 mM) concentrations of Etn (Fig. 3). Since HC-3 competitively inhibits high-affinity Cho transport (but not Etn transport) and kinase-mediated phosphorylation of Cho, we considered the possibility that HC-3 and Cho may oppositely regulate the promitogenic effect of Etn. Indeed, as shown in Fig. 3, HC-3 (1 mM) slightly inhibited the combined actions of Etn and insulin on DNA synthesis. Surprisingly, the inhibitory effects of HC-3 were enhanced further by 40 mM EtOH (Fig. 3). Presently, we have no clear explanation of why EtOH should enhance the inhibitory effect of HC-3. However, we should note that in this experiment (Fig. 3) HC-3 was used at a relatively low (1 mM) concentration (which, however, still strongly inhibited both Cho uptake and Cho phosphorylation; see Fig. 7), because at higher concentrations it enhanced the effect of insulin on DNA synthesis to an extent that interfered with its inhibition of promitogenic Etn effects. Thus, it is an experimentally testable possibility that at these suboptimal concentrations EtOH actually enhanced the interaction of HC-3 with the Cho site.

The above experiments presented thus far suggested that even if EtOH may not directly interact with an Etn binding site, it still may affect a secondary site through which both Cho (as an agonist) and HC-3 (as an antagonist) are capable of modifying the promitogenic effect of Etn. If this is the case, then one would expect that both Cho and higher concentrations of HC-3 will inhibit the promitogenic effect of EtOH. As shown in Fig. 4, although both Cho (5 mM) and HC-3 (3 mM) actually doubled the small individual effect of insulin on DNA synthesis (for which the reason is presently unknown), these compounds indeed partially inhibited the synergistic effects of insulin and 80 mM EtOH.

Effects of EtOH on Cellular Binding/Uptake and Metabolism of Radiolabeled Cho and Etn

We hypothesized that if EtOH interacts with either an Etn-binding or Cho-binding site in the cell membrane, then EtOH would be expected to inhibit binding of these compounds to membranes. However, the predictably very low affinity of these compounds to their hypothetical

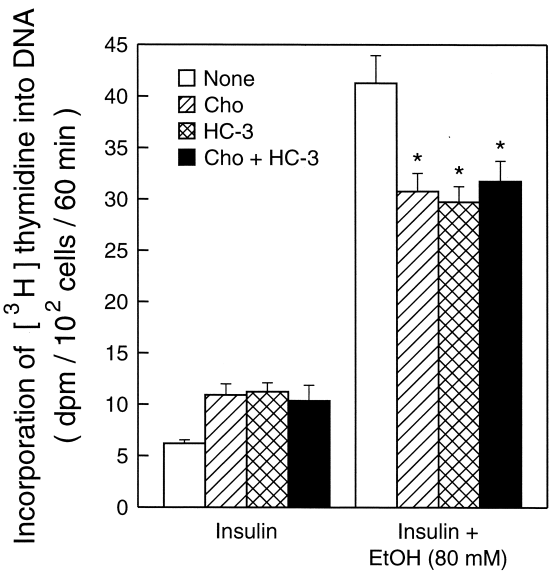

FIG. 4. Partial inhibition by Cho and HC-3 of the synergistic stimulatory effects of EtOH and insulin on DNA synthesis. Serum-starved NIH 3T3 fibroblasts were treated with 25 μM zinc for 20 min, followed by incubations first for 30 min in the absence or presence of 5 mM Cho, 3 mM HC-3, or Cho plus HC-3 and then for 18 hr in the absence or presence of 500 nM insulin as indicated. Data are the means ± SEM of three experiments, each performed in triplicate. Key: (*) significantly ($P < 0.01$) different from the combined effects of insulin and EtOH.

binding sites (judged from the high concentrations required for their effects on DNA synthesis) raises the possibility that technically one might not be able to measure such binding. Indeed, employing a plasma membrane-enriched fraction which we previously used to characterize phospholipase D activity [38], we were unable to show significant binding of [^{14}C]Etn to membranes by a filtration method. Apparently, washing of membranes with a large volume of buffer was sufficient to fully dissociate Etn from the membrane, if such binding occurred at all.

Binding of Etn to cell membrane may be somewhat stronger in intact cells. In addition, washing of cells in a well is a less drastic procedure than washing of membranes over a filter; this might further help to preserve some Etn binding in intact cells. Therefore, next we attempted to demonstrate an effect of EtOH on the amount of cell-associated [^{14}C]Etn during a shorter (10 min) and a longer (40 min) incubation period. The idea was that if EtOH were to reduce the amount of cell-associated [^{14}C]Etn without affecting the formation of [^{14}C]PEtn and [^{14}C]PtdEtn, this would indicate that EtOH affected cell membrane binding but not cellular uptake of [^{14}C]Etn. To decrease the background values caused by [^{14}C]Etn uptake (versus presumed binding), we also employed high concentrations of unlabeled Cho, which we expected to specifically decrease cellular uptake but not binding of [^{14}C]Etn. Clearly, over a 10-min period, 80 mM EtOH did not have any detectable effects on the cellular content of [^{14}C]Etn, [^{14}C]PEtn, or [^{14}C]PtdEtn (Fig. 5). In another experiment, EtOH also had no effects on the amount of cell-associated [^{14}C]Etn in the presence of 15 μM zinc (data not shown). As an additional interesting observation, after a 10-min incubation Cho appeared to have greater inhibitory effects on cell association of [^{14}C]Etn than on the synthesis of [^{14}C]PEtn or [^{14}C]PtdEtn (Fig. 5).

When the incubation time was 40 min instead of 10 min, in the absence of choline, EtOH slightly, but significantly, decreased the amount of cell-associated [^{14}C]Etn (Fig. 6). An inhibitory effect of EtOH also was seen in the presence of a 0.25 mM, but not a 1 or 5 mM, concentration of Cho (Fig. 6). Again, the addition of 15 μM zinc did not modify the effects of EtOH significantly (not shown). Interestingly, at the 40-min time point, Cho had smaller inhibitory effects on cell association of [^{14}C]Etn (Fig. 6) compared with its effects after the 10-min incubation (Fig. 5), and it had no inhibitory effects on [^{14}C]PtdEtn synthesis at all (Fig. 6). Although we cannot prove it at this point, these data may mean that initially the Etn binding site is occupied by an endogenous ligand that is slowly replaced by [^{14}C]Etn; as a result, over time the binding component would gradually represent a greater fraction of total cell-associated [^{14}C]Etn, which now would be more sensitive to EtOH inhibition and less sensitive to Cho inhibition. It is still important to note that the inhibitory effect of EtOH on cell association of [^{14}C]Etn was not accompanied by inhibition of [^{14}C]PEtn or [^{14}C]PtdEtn synthesis (Fig. 6). This clearly indicates that EtOH affected the association of

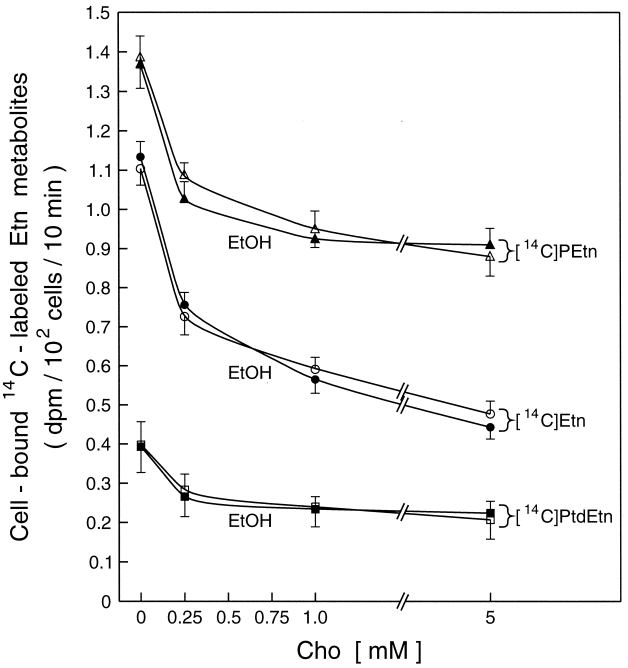

FIG. 5. Effects of EtOH on the cellular content of [^{14}C]Etn and its metabolites during shorter-term incubation. Serum-starved NIH 3T3 fibroblasts were incubated for 10 min with [^{14}C]Etn in the presence of 0–5 mM Cho, as indicated, in the absence (open symbols) or presence (closed symbols) of 80 mM EtOH. [^{14}C]Etn and its metabolites were determined as described in Materials and Methods. Each point represents the mean ± SD of six separate samples in a single experiment.

[^{14}C]Etn with a cellular pool that is not involved in metabolism and, therefore, may not even be located inside the cell.

The inability of EtOH to inhibit Etn binding at the earlier time point (Fig. 5) and in the presence of 1–5 mM Cho at the later time point (Fig. 6) could also be an indication that EtOH in fact primarily inhibited Cho binding to cells. Indeed, 80 mM EtOH slightly (by ~18%) but significantly decreased association of [^{14}C]Cho with the cells (Fig. 7). In another experiment, 15–25 μM zinc did not alter this inhibitory EtOH effect (not shown). HC-3, an inhibitor of both Cho uptake and (presumably) Cho binding, strongly inhibited cell association of [^{14}C]Cho and it also diminished the inhibitory effect of EtOH (Fig. 7). EtOH had no statistically significant inhibitory effects on [^{14}C]PCho formation in the absence or presence of HC-3 (Fig. 7). Incorporation of [^{14}C]Cho into PtdCho was too small at the 10-min time point for a proper evaluation.

DISCUSSION

The major conclusion of this work is that the previously observed promitogenic effect of EtOH is mimicked, to varying extents, only by MeOH and Etn, but not by 3- to 9-carbon normal alcohols. In view of this specificity, it seems likely that EtOH may exert its effects by interacting with a small hydrophobic pocket of a receptor-like protein.

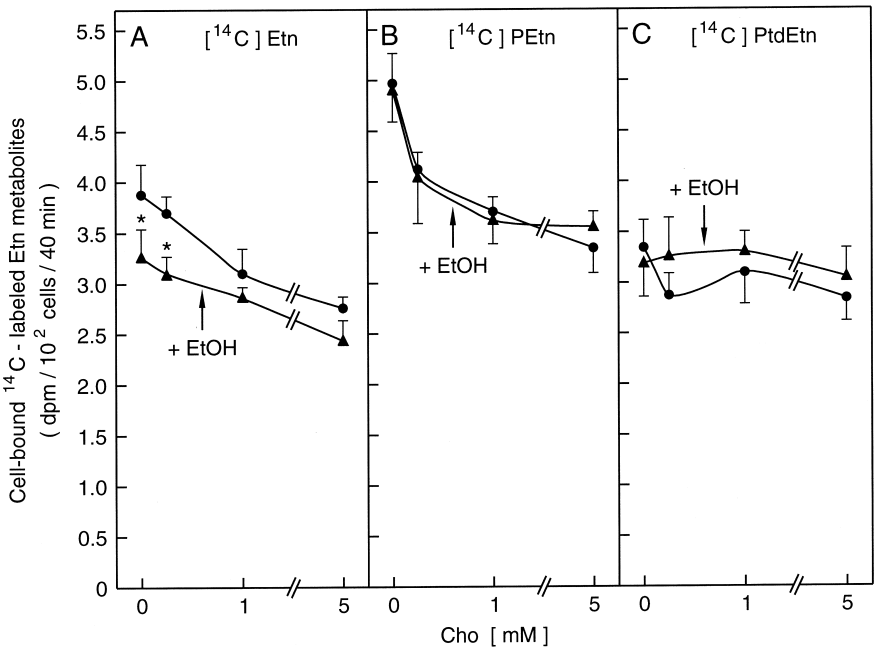

FIG. 6. Effects of EtOH on the amount of cell-associated [^{14}C]Etn during longer-term incubation. Serum-starved NIH 3T3 fibroblasts were incubated for 40 min with [^{14}C]Etn in the presence of 0–5 mM choline, as indicated, in the absence (●) or presence (▲) of 80 mM EtOH. Cell-associated [^{14}C]Etn (A), [^{14}C]PEtn (B), and [^{14}C]PtdEtn (C) were determined as described in Materials and Methods. Each point represents the mean ± SEM of three experiments, each performed in triplicate. Key: (*) significantly ($P < 0.05$) smaller than the corresponding value in the absence of EtOH.

In fact, it has been shown before that various membranes have saturable EtOH binding sites [36, 39, 40], which is consistent with the idea that EtOH can bind, although weakly, to certain proteins. It is interesting to note that in some other systems as well, higher carbon number alcohols do not tend to reproduce the effects of EtOH. For example, whereas it has been shown that MeOH, EtOH, and *n*-propanol each can enhance the function of the G-protein-coupled inwardly rectifying potassium channels via interacting with a specific region in the carboxyl terminus, longer-chain alcohols have no effects [41, 42]. Similarly, only the above short-chain alcohols, but not longer-chain normal alcohols, are able to modify the activity of membrane-bound acetylcholinesterase [43].

An important aspect of the actions of EtOH and MeOH is that they enhanced insulin-stimulated DNA synthesis only if no attempt was made to prevent their evaporation from the wells. This implies that an early stimulatory alcohol action is followed by an inhibitory phase that can negate the stimulatory action if slow evaporation of alcohols is not allowed to take place. The temporal relationship between the stimulatory and inhibitory actions of EtOH on DNA synthesis in fibroblasts and other cell types will be treated in detail elsewhere.

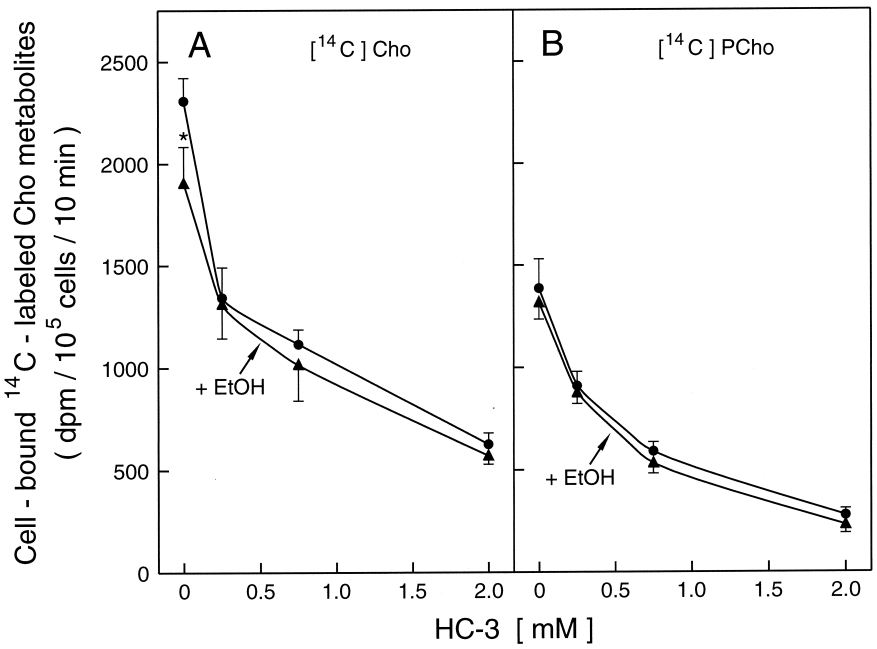

FIG. 7. Effects of EtOH on the amount of cell-associated [^{14}C]Cho during shorter-term incubation. Serum-starved NIH 3T3 fibroblasts were incubated for 10 min with [^{14}C]Cho in the presence of 0–2 mM HC-3, as indicated, in the absence (●) or presence (▲) of 80 mM EtOH. Cell-associated [^{14}C]Cho (A) and [^{14}C]PCho (B) were determined as described in Materials and Methods. Each point represents the mean ± SEM of three experiments, each performed in triplicate. Key: (*) significantly ($P < 0.05$) smaller than the corresponding value in the absence of EtOH.

Concerning the site and mechanism of EtOH action, our results suggest that there is a complex relationship between the effects of EtOH, Etn, and Cho on insulin-stimulated DNA synthesis. Although concomitant cellular uptake of Etn and Cho makes it extremely difficult to determine their possible low-affinity binding to the cell membrane, our data clearly indicate that EtOH can decrease the total amount of cell-associated Cho and, after longer incubations, Etn. Based on these findings, we hypothesize that EtOH acts on mitogenesis via a membrane site for which Cho is an agonist and HC-3 an antagonist. In addition, the data do not exclude the possibility that EtOH also interacts with an Etn binding site that may also be able to bind an endogenous ligand.

Importantly, the effects of EtOH on the cell association of Cho and Etn were not affected by zinc, suggesting that zinc is required at a later step, perhaps to specifically prevent the inhibitory effect of EtOH on MAP kinase activation [31]. Since Etn does not inhibit MAP kinase stimulation by insulin (Mukherjee JJ and Kiss Z, unpublished observations), this may explain why the promitogenic effect of Etn does not require zinc. Another difference between the actions of Etn and EtOH is that we have not been able to detect, thus far, an inhibitory action by the former (Crilly KS and Kiss Z, unpublished observations).

In summary, our results suggest that the promitogenic actions of EtOH involve Cho and perhaps Etn binding sites. However, to provide a final proof for this possibility, it will be necessary to develop high-affinity, biologically active ligands that selectively interact with these presumed sites. Such work is presently underway in this laboratory.

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