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Influence of Acute and Chronic Ethanol Treatment on Muscarinic Responses and Receptor Expression in Chinese Hamster Ovary Cells

*Zong Liang Chang, Henry L. Puhl, Lisa G. May, Carol L. Williams and Robert S. Aronstam**

GUTHRIE RESEARCH INSTITUTE, SAYRE, PA 18840, U.S.A.

ABSTRACT. The influence of ethanol on the muscarinic receptor-mediated release of inositol phosphate from Chinese hamster ovary (CHO) cells stably transfected with one of the five subtypes of muscarinic acetylcholine receptor was determined. In CHO cells expressing M3 muscarinic receptors (CHO-M3), carbamylcholine increased muscarinic receptor-induced release of inositol phosphate by 150–350% following a 15-min incubation with an EC_{50} of ≈30 μM. Maximal responses were obtained with 1 mM carbamylcholine, while responses to 10 mM carbamylcholine were somewhat less than maximal. Preincubation with atropine for 10 min inhibited the response with an IC_{50} of ≈30 nM. CHO cells transfected with M1, M3, and M5 receptors displayed a similar pattern of activity; CHO cells transfected with M2 and M4, as well as untransfected cells, were unresponsive to carbamylcholine. Ethanol acutely inhibited the response of CHO-M3 cells to carbamylcholine by 15% at 18 mM and by 47% at 180 mM (the highest concentration examined). CHO-M3 cells were incubated with 50 mM ethanol for 48 hr. This treatment did not affect the number of cells or their protein content (113 pg/cell). The expression of M3 muscarinic receptors (determined using [^{3}H]*N*-methylscopolamine) increased from 1.34 ± 0.23 to 1.75 ± 0.16 pmol/mg protein ($P < 0.05$). In contrast, carbamylcholine-stimulated release of inositol phosphate was depressed by 40–70% in four experiments. Concentration–response analyses indicated a non-competitive inhibitory mechanism. This dissociation of muscarinic receptor expression and muscarinic signaling suggests a compensatory increase in receptor expression in response to chronic inhibition of muscarinic signaling by ethanol. BIOCHEM PHARMACOL **54**;7:833–839, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ethanol; muscarinic receptors; signal transduction; phosphatidylinositol; phospholipase C; CHO cells

Ethanol has a wide range of effects on intercellular signaling functions in the central nervous system [1–4]. In general, lower concentrations of ethanol are required to inhibit synaptic transmission than are required to disrupt the electrical properties of nerve cells (e.g. [5]). Accordingly, recent attention in ethanol research has focused on a range of synaptic mechanisms, including γ-aminobutyric $acid_A$, glutamate (especially *N*-methyl-D-aspartate), neuropeptide and adenosine receptors, chemically- and voltage-gated ion channels, transducer G proteins, and second messenger pathways [6].

Acute ethanol administration causes a decrease in acetylcholine release from brain tissue *in vivo* and *in vitro* [7–9], as well as alterations in brain acetylcholine levels and turnover [10, 11]. Several acute effects of ethanol on postsynaptic muscarinic processes have also been described [1]. Ethanol selectively enhances the sensitivity of cholinergic transmission in the hippocampus [12, 13]. Ethanol inhibits antagonist binding to muscarinic receptors, but only at very high concentrations (IC_{50} values > 1 M), and the potency of short-chain alcohols at inhibiting antagonist binding is related directly to the length of the carbon chain [14]. At physiologically tolerable concentrations, ethanol selectively decreases agonist binding affinity to muscarinic receptors by decreasing hydrophobic binding effects [15, 16]. Ethanol has also been reported to inhibit muscarinic receptor-mediated stimulation of [^{32}P] incorporation into synaptosomal phosphatidic acid [17], but not muscarinic receptor-mediated inhibition of adenylate cyclase activity in the striatum [18]. Ethanol preferentially inhibits muscarinic activation of phosphoinositide metabolism in neonatal rats [19]. Larsson *et al.* [20] demonstrated that ethanol disrupts muscarinic stimulation of phosphoinositide metabolism in SH-SY5Y human neuroblastoma cells in a protein kinase C-dependent manner. Similarly, work by Sanna *et al.* [21] led to the suggestion that ethanol activates protein kinase C which phosphorylates M1 receptors, thereby depressing the ability of acetylcholine to activate phosphoinositide metabolism.

Effects on G proteins have been implicated in the synaptic actions of ethanol. We previously demonstrated

* Corresponding author: Dr. Robert S. Aronstam, Guthrie Research Institute, One Guthrie Square, Sayre, PA 18840. Tel. (717) 882-4624; FAX (717) 882-5151; E-mail: raronsta@inet.guthrie.org.

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that ethanol (1) alters ligand binding to the muscarinic acetylcholine receptor, and (2) alters receptor coupling to guanine nucleotide-dependent transducer proteins (G proteins) [22]. Ethanol increases the activation of adenylate cyclase by G_s (e.g. [23, 24]). Hoffman and Tabakoff [25] have proposed that ethanol selectively enhances the rate of activation of G_s (an action that is normally catalyzed by an interaction with receptors) as well as the interaction of $G_{\alpha s}$ with guanine nucleotides. On the other hand, Bauché *et al.* [26] have presented evidence that ethanol also disrupts G_i-mediated control of adenylate cyclase in rat brain. Charness *et al.* [27] have demonstrated differential regulation by ethanol of the expression of both $G_{i\alpha}$ and $G_{s\alpha}$ in several neuronal cell lines.

In the present study, the effects of ethanol on muscarinic receptor-mediated increases in phosphatidylinositol metabolism were investigated using CHO† cells stably transfected with specific subtypes of muscarinic receptor. Ethanol was found to inhibit acutely muscarinic-mediated inositol phosphate generation. Exposure of the cells to ethanol for 2 days decreased muscarinic signaling through this pathway that was accompanied by an increase in the expression of muscarinic receptors. This suggests that a chronic inhibition of this pathway leads to a compensatory up-regulation of receptor expression.

MATERIALS AND METHODS

CHO cells stably transfected with one of the five subtypes of human muscarinic acetylcholine receptor were provided by Dr. Mark R. Brann (University of Vermont). The cells were cultured in HAM's F-12 with 5% fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM), and grown in 150 cm^2 flasks at 37°/5% CO_2.

Inositol Phosphate Release

Muscarinic receptor control of phospholipase C activity was monitored by measuring agonist-mediated release of [^{3}H]inositol phosphate from cells prelabeled with [^{3}H]inositol, using a modification of the methods of Claro *et al.* [28]. CHO cells (6×10^5/mL) were labeled with 2.5 μCi/mL [^{3}H]*myo*-inositol (Dupont-NEN, Boston, MA) for 4 hr. The cells were washed twice in serum-free medium and resuspended in the same medium at a concentration of 1.5×10^6 cells/0.25 mL. The cells were preincubated with 20 mM lithium chloride for 20 min at 37°/5% CO_2 followed by incubation with 100 μM carbamylcholine for 15 min or the indicated time. The reaction was terminated by the addition of 0.22 M HCl (final concentration, 18 mM). Water-soluble metabolites were extracted with chloroform:methanol (0.54 mL; 1:2, v/v), followed by separation of the phases with equal amounts (0.18 mL) of chloroform and water. After centrifugation at 1000 *g* for 10 min, the aqueous phase was collected, and radioactivity content was determined by liquid scintillation counting.

To determine the effect of ethanol on muscarinic receptor-mediated [^{3}H]inositol phosphate release, prelabeled cells were exposed to ethanol for 10 min prior to the addition of carbamylcholine. To determine the effect of chronic exposure to ethanol on muscarinic receptor-mediated [^{3}H]inositol phosphate release, CHO cells were incubated with 50 mM ethanol for 48 hr, changing the medium after 24 hr. Control cells were treated in a similar manner without the ethanol. The cells were then labeled with [^{3}H]*myo*-inositol in the continued presence of ethanol, and muscarinic receptor-mediated [^{3}H]inositol phosphate release was measured as described above.

Receptor Measurements

The content of muscarinic acetylcholine receptors in the cells was measured using the radiolabeled antagonist [^{3}H]MS (Dupont-NEN). The cells were harvested by scraping and homogenized in 50 mM Tris–HCl, pH 7.4. Protein content was determined by the BCA assay kit (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the standard. The binding medium contained 15–30 μg protein, [^{3}H]MS, and 50 mM Tris–HCl, pH 7.4. Nonspecific binding was determined in the presence of 10 μM

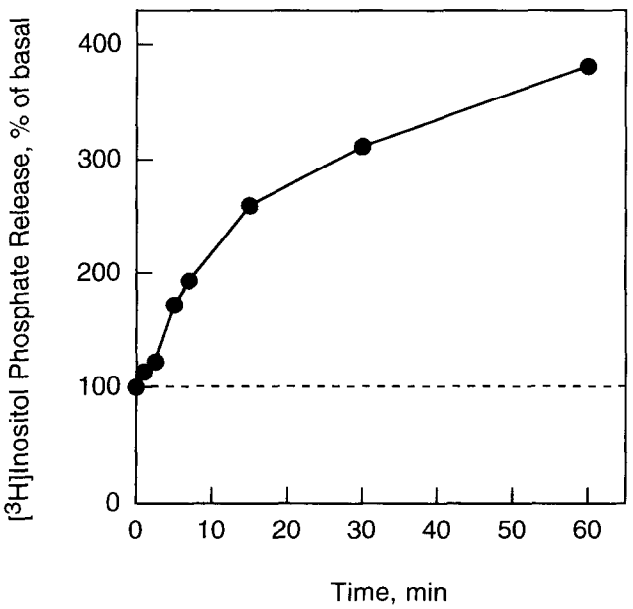

FIG. 1. Time–course for carbamylcholine stimulation of [^{3}H]inositol phosphate release from CHO cells transfected with the human M3 muscarinic receptor. [^{3}H]Inositol phosphate release was measured at the indicated times after the addition of 100 μM carbamylcholine. Release is expressed as a percent of basal release measured in the absence of carbamylcholine. Each value represents the mean from 3 experiments, which varied by less than 15%. The extent of labeling with [^{3}H]inositol was 2.91 ± 0.18 pmol/mg cellular protein (N = 6); basal release averaged 10.5 ± 2.6%.

† *Abbreviations:* CHO, Chinese hamster ovary; and [^{3}H]MS, [^{3}H]*N*-methylscopolamine.

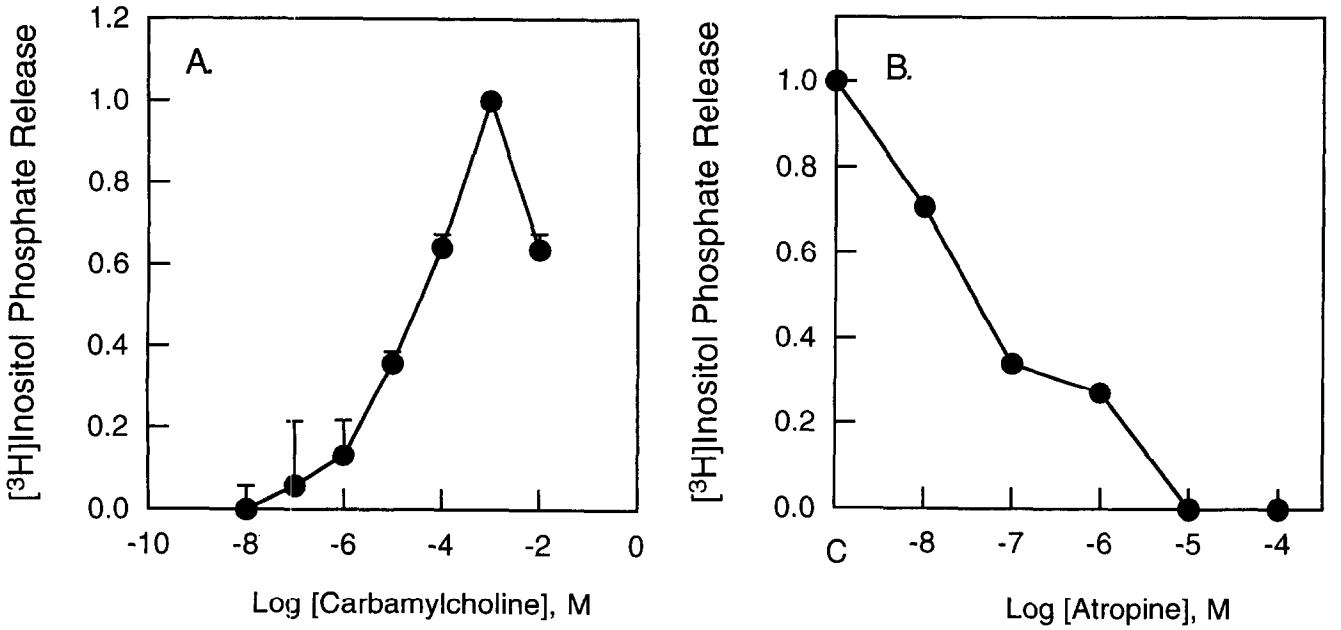

FIG. 2. Carbamylcholine stimulation of [^{3}H]inositol phosphate release from CHO cells transfected with the M3 muscarinic receptor: concentration–response curve and atropine sensitivity. (A) [^{3}H]Inositol phosphate release was measured after a 15-min incubation in the presence of the indicated concentration of carbamylcholine. Release is expressed as a fraction of the maximal release obtained with the maximally effective concentration of carbamylcholine (1 mM). Each value represents the mean ± SD from 3–5 experiments. The mean maximal release was 420% of basal release measured in the absence of carbamylcholine. The extent of labeling with [^{3}H]inositol in this series of experiments was 1.31 ± 0.09 pmol/mg protein, basal release averaged 5.5 ± 0.3%, and the maximal stimulated release represented 32.2 ± 10.6% of the total label. (B) [^{3}H]Inositol phosphate release was measured after a 15-min incubation in the presence of 100 μM carbamylcholine. Atropine was added 10 min before the carbamylcholine. Release is expressed as a fraction of maximally stimulated release. Each value represents the mean from 3 experiments, which varied by less than 15%.

unlabeled MS. The suspension was incubated for 90 min at room temperature, and the binding was determined by filtration through glass fiber filters. Binding measurements were performed using seven concentrations of [^{3}H]MS, and saturation curves were analyzed by non-linear regression to determine receptor concentration and ligand dissociation constant.

RESULTS

The time–course of carbamylcholine-stimulated release of [^{3}H]inositol phosphate from cultured CHO-M3 cells is illustrated in Fig. 1. The total release increased for at least 60 min with all receptor subtypes (data not shown), and 15 min was used as the time point for all subsequent experiments.

The concentration-dependence for carbamylcholine-stimulated [^{3}H]inositol phosphate release from CHO-M3 cells is depicted in Fig. 2A. A maximal response required 1 mM carbamylcholine; at higher concentrations, [^{3}H]inositol phosphate release was reduced. The EC_{50} for carbamylcholine-stimulated release was approximately 30 μM. Preincubation for 10 min with atropine inhibited the response of CHO-M3 cells to carbamylcholine with an IC_{50} of about 30 nM (Fig. 2B).

Responses of CHO cells transfected with each of the five receptor subtypes is shown in Fig. 3, A and B. Only the cells transfected with M1, M3, or M5 receptors responded to carbamylcholine stimuation with an increase in [^{3}H]inositol phosphate release, in agreement with the preferential coupling of the odd-numbered receptors to phospholipase C through transducer proteins of the G_q-class [29]. Cells transfected with M2 or M4 receptors, or transfected with the vector only (M0), displayed no increase in [^{3}H]inositol phosphate release in response to carbamylcholine (Fig. 3).

The concentration-dependence for acute ethanol inhibition of carbamylcholine-stimulated [^{3}H]inositol phosphate release from CHO-M3 cells is depicted in Fig. 4. Inhibition was obtained with ethanol concentrations as low as 18 mM; 47% inhibition was observed in the presence of 180 mM ethanol.

To understand the long-term effects of ethanol on muscarinic signaling processes, CHO-M3 cells were incubated with 50 mM ethanol for 48 hr. Then the cells were harvested for determination of phospholipase C activity and receptor content. The number of cells in the cultures was not affected by treatment for 48 hr with 50 mM ethanol, nor was the protein content of each cell (113 pg protein/cell). In a series of four experiments, the responses to carbamylcholine were inhibited (Fig. 5A); the magnitude of the responses were depressed rather than the

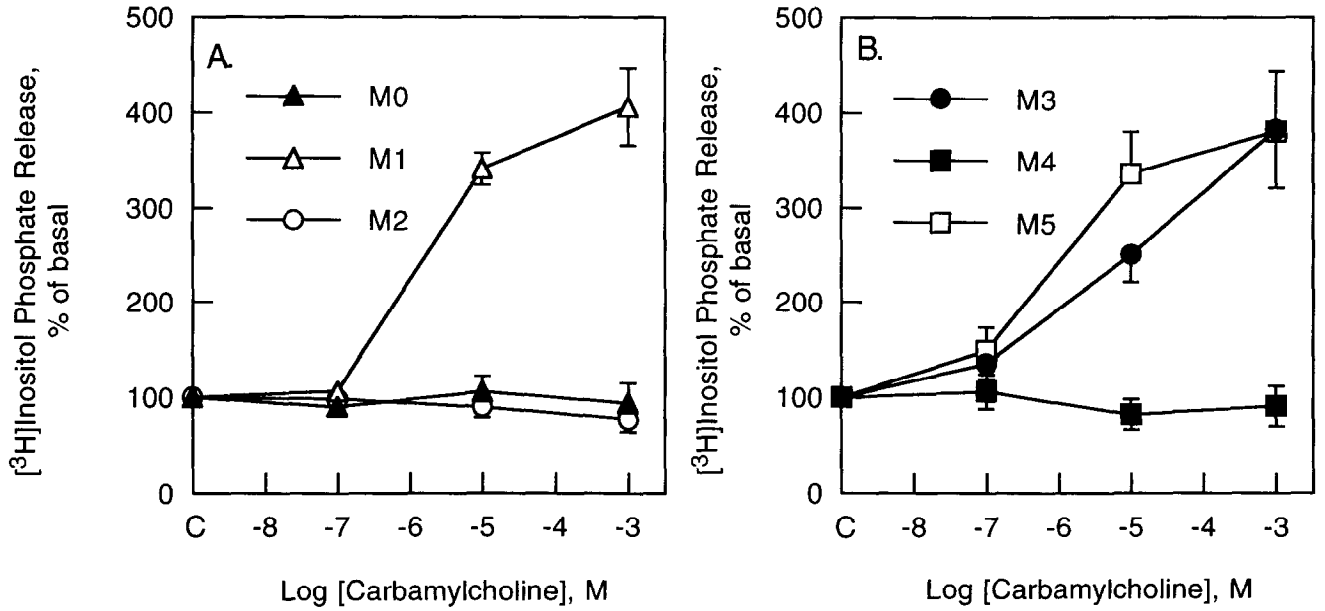

FIG. 3. Carbamylcholine stimulation of [^{3}H]inositol phosphate release from CHO cells transfected with different subtypes of muscarinic acetylcholine receptor. CHO cells were stably transfected with the M1, M2, M3, M4, or M5 muscarinic receptors, as indicated, or were untransfected (M0). [^{3}H]Inositol phosphate release was measured after a 15-min incubation in the presence of 100 μM carbamylcholine. Release is expressed as a percent of basal release measured in the absence of carbamylcholine. Each value represents the mean ± SD from 3 experiments. The extent of labeling of the various cells was as follows (all values are in pmol/mg cellular protein, and the fraction of basal release is indicated in the parentheses; N = 3): M0, 2.76 ± 0.08 (6.9 ± 0.5%); M1, 1.93 ± 0.13 (6.9 ± 0.7%); M2, 1.76 ± 0.20 (6.8 ± 0.3%); M3, 1.93 ± 0.14 (6.5 ± 1.6%); M4, 1.85 ± 0.31 (6.8 ± 0.4%); and M5, 2.55 ± 0.75 (6.7 ± 0.75%).

sensitivity to carbamylcholine ($EC_{50} \approx 10$ μM in both groups). Basal [^{3}H]inositol phosphate release was not affected by ethanol treatment (7.0 ± 1.6 and 7.9 ± 1.7% of the labeled pool in the ethanol-treated and untreated groups, respectively; mean ± SD, N = 4). The lack of an effect on basal release indicates that ethanol did not affect *myo*-inositol incorporation under these conditions. The concentration dependence of the chronic inhibition is depicted in Fig. 5B. The response to carbamylcholine was 22% lower after exposure to 5 mM ethanol for 48 hr, and 44% lower after prolonged exposure to 50 mM ethanol.

The influence of chronic (48-hr) exposure of CHO-M3 cells to 50 mM ethanol on the expression of M3 receptors was determined to evaluate the contribution of receptor expression to altered muscarinic responsiveness. The affinity of the receptors for [^{3}H]MS was not affected by the ethanol treatment. The density of receptors, however, was increased from 1.34 ± 0.23 to 1.75 ± 0.16 pmol/mg protein (mean ± SD, N = 4; $P < 0.05$, Student's *t*-test) (inset, Fig. 5B). Insofar as the number of cells and the protein content of the cells were not altered by the ethanol treatment (113 pg/cell), the density of receptor expression per cell was increased to a similar extent (31%). These muscarinic receptor measurements were performed using aliquots from the same four groups of cells that were used to determine the effects of chronic ethanol treatment on carbamylcholine-stimulated [^{3}H]inositol phosphate release depicted in Fig. 5A.

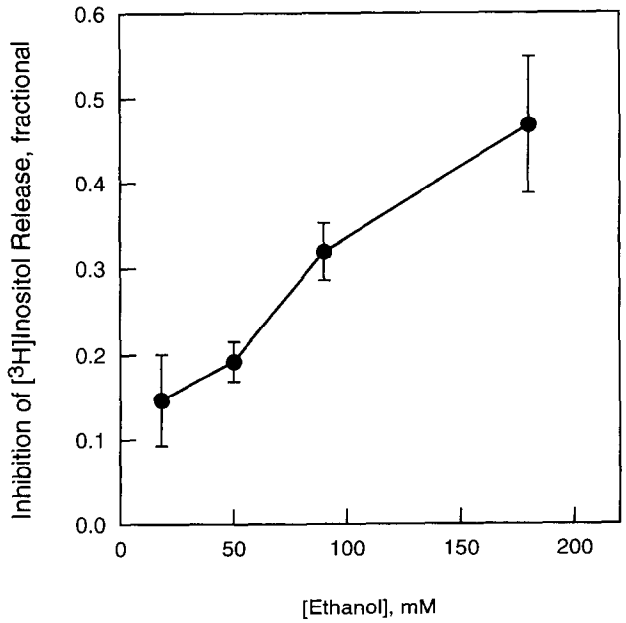

FIG. 4. Influence of ethanol on muscarinic receptor-mediated release of [^{3}H]inositol phosphate from CHO cells transfected with the M3 subtype of muscarinic acetylcholine receptor. Ethanol was added to the cells 10 min before [^{3}H]inositol phosphate release was assayed. Release was measured 15 min after the addition of 100 μM carbamylcholine. The fractional inhibition of [^{3}H]inositol phosphate release is plotted as a function of ethanol concentration. Each value represents the mean ± SD from 3 experiments. The extent of labeling of the cells was 2.93 ± 0.17 pmol/mg protein.

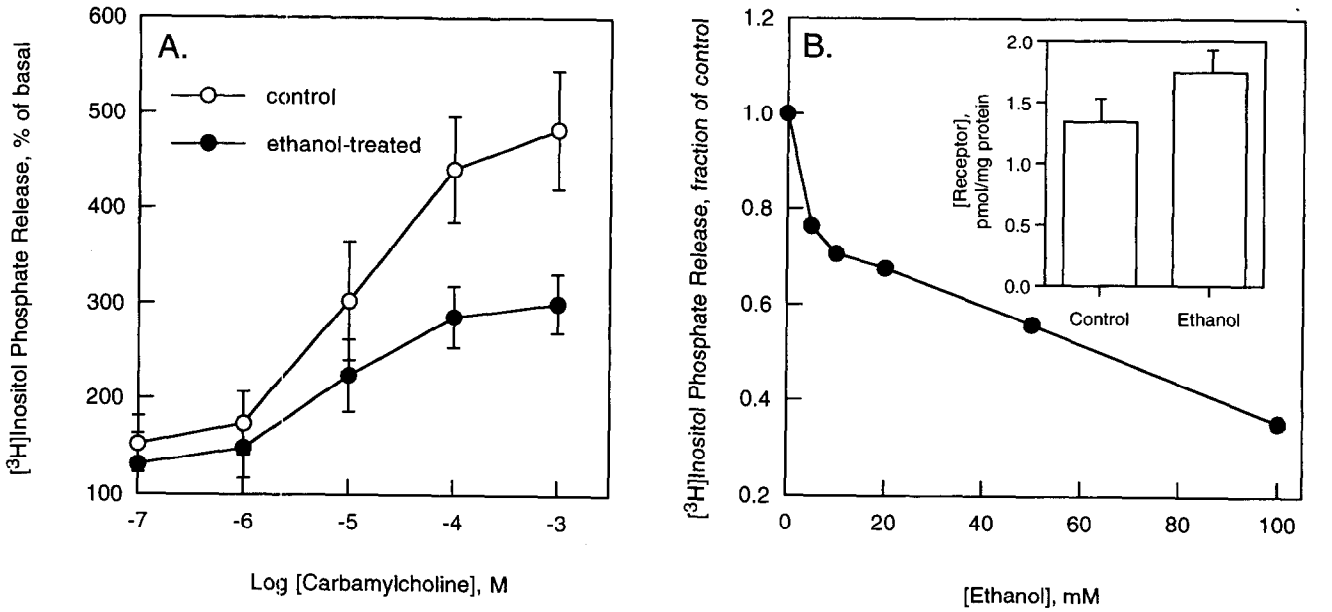

FIG. 5. Influence of chronic ethanol treatment on muscarinic receptor-mediated release of [^{3}H]inositol phosphate from CHO cells transfected with the M3 subtype of muscarinic acetylcholine receptor. (A) Cells were incubated with 50 mM ethanol for 48 hr before being loaded with [^{3}H]*myo*-inositol in the continued presence of ethanol (●); control cells were treated in an identical manner in the absence of ethanol (○). Then the cells were washed and prepared for [^{3}H]inositol phosphate release measurements performed in the absence of ethanol. Release was measured after a 15-min incubation in the presence of the indicated concentration of carbamylcholine, and is expressed as a percent of [^{3}H]inositol phosphate release measured in the absence of carbamylcholine. Each value represents the mean ± SD from 4 experiments. The extent of labeling of the control cells was 1.72 ± 0.26 pmol/mg protein and basal release was 6.2 ± 1.2%; the extent of labeling of the ethanol-treated cells was 1.82 ± 0.33 pmol/mg protein and basal release was 8.8 ± 2.4%. (B) Cells were incubated for 48 hr in the presence of the indicated concentrations of ethanol. The cells were then washed and prepared for [^{3}H]inositol phosphate release measurements performed in the absence of ethanol. Release was measured after a 15-min incubation with 100 μM carbamylcholine. Release is expressed as a fraction of release from cells not exposed to ethanol. Each value represents the mean from 3 experiments, which varied by less than 15%. (Inset, Fig. 5B) Influence of chronic ethanol treatment on the expression of muscarinic receptors by CHO cells transfected with the M3 subtype of human muscarinic acetylcholine receptor. Cells were incubated with 50 mM ethanol for 48 hr before being loaded with [^{3}H]*myo*-inositol in the continued presence of ethanol (Ethanol); control cells were treated in an identical manner in the absence of ethanol (Control). Receptor concentration was measured using [^{3}H]MS as the probe. Each value represents the mean ± SD from 4 experiments performed in quadruplicate. The level of receptor expression was significantly higher ($P < 0.05$; Student's two tailed *t*-test) in ethanol-treated CHO cells.

DISCUSSION

In the present experiments, ethanol was found to inhibit acutely M3 muscarinic receptor-induced release of inositol phosphate from CHO cells by up to 47%. Exposure of CHO-M3 cells to 50 mM ethanol for 48 hr decreased muscarinic stimulation of inositol phosphate release but increased the expression of M3 receptors by 31%. Thus, there is a dissociation of muscarinic responsiveness and receptor expression as a consequence of prolonged exposure of cultured cells to ethanol.

The expression of muscarinic (and most other) receptors is affected by the degree of receptor activation. Thus, chronic exposure to receptor agonists decreases receptor expression (down-regulation), while chronic inhibition of the receptors increases receptor expression (up-regulation). Nicotinic acetylcholine receptors in the brain up-regulate in response to chronic exposure to both agonists and antagonists, probably reflecting the rapid agonist-induced desensitization that is characteristic of these receptors (e.g. [30]). Similarly, sensitization of signal transduction pathways, such as that seen in disease states (e.g. diabetes [31]), is accompanied frequently by a decrease in receptor expression. These processes may reflect the homeostatic tendency of cells to maintain a constant level of external stimulation. The dissociation of receptor signaling and expression suggests that the increase in expression upon chronic exposure to ethanol is in response to a functional inhibition of muscarinic signaling.

Ethanol disrupts most metabolic processes when presented at a sufficiently high concentration; therefore, we are particularly interested in the processes that are most sensitive to its presence. The behavioral effects of ethanol are due to a depression of neuronal function, and synaptic communication between neurons is more sensitive to disruption by ethanol than is axonal conduction (e.g. [5]). The precise site(s) of this disruption is not clear. Concentrations of ethanol associated with impairment of reaction time in humans are about 4–6 mM; concentrations associated with gross intoxication range from 30 to 50 mM, and lethal concentrations are generally above 80 mM [32]. In the present experiments, muscarinic receptor-stimulated release of inositol phosphate was inhibited acutely by ethanol

concentrations as low as 18 mM, while chronic inhibition was observed with ethanol concentrations as low as 5 mM. These results suggest that this action may contribute to physiological disturbances in intoxication.

A central question addressed by the present study is the relationship of muscarinic receptor expression to muscarinic neurotransmission, and how this relationship is affected by ethanol. We observed a decrease in functional transmission (i.e. muscarinic control of phosphoinositide metabolism) that was accompanied by an increase in receptor expression. Larsson *et al.* [33] recently described the effects of exposure of human neuroblastoma cells (SH-SY5Y) to ethanol on M1 muscarinic receptor stimulation of phospholipase C activity. Exposure to 100 mM ethanol for 4 days caused an *increase* in muscarinic receptor-stimulated release of inositol 1,4,5-trisphosphate that was accompanied by an increase in muscarinic receptor expression. The reason for the discrepancy with the present results (i.e. the increase rather than a decrease in muscarinic control of phosphoinositide metabolism) is unclear, although it may reflect the use of different cells, ethanol concentration, and exposure period. There is a widespread decrease in the concentration of muscarinic binding sites in the brains of human alcoholics at autopsy, although the relationship of this change to functional muscarinic transmission is uncertain. Frye *et al.* [34] failed to observe a change in physiological muscarinic transmission in the hippocampi of rats treated with ethanol for up to 3–6 months.

A different relationship between muscarinic receptor density and control of phosphoinositide metabolism is observed during the natural dissociation of receptor and signal pathway expression that occurs during development. Muscarinic receptor-mediated stimulation of phosphoinositide metabolism is extremely sensitive to ethanol in neonatal mice and rats, a time when there is a very efficient coupling of muscarinic receptors to phosphoinositide metabolism, but receptor density is low [35]. Two months later, the number of muscarinic receptors has doubled while the functional coupling of receptor to phosphoinositide metabolism and the sensitivity of this coupling to ethanol have declined markedly [35]. These divergent relationships between receptor expression and transmission potential and sensitivity to ethanol suggest multiple sites of ethanol action. Moreover, it seems likely that structures other than the receptor and phospholipase C are affected by ethanol.

In summary, muscarinic receptor-stimulated release of inositol phosphate was found to be depressed by chronic exposure to 50 mM ethanol in CHO cells expressing human M3 muscarinic receptors. The variable relationship between receptor expression, muscarinic control of phosphoinositide metabolism, and the sensitivity of this signaling pathway to ethanol suggests that the receptors are not the primary site of ethanol action. This conclusion is further supported by the minimal acute effects of ethanol on muscarinic binding activities and the recent demonstrations of the protein kinase C dependence of ethanol inhibition of muscarinic transmission [20, 24]. Ethanol disruption of muscarinic transmission may involve alteration of receptor-G protein coupling interactions or effector and transducer phosphorylation processes.

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