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Acute Ethanol Treatment Modulates δ Opioid Receptors in N18TG2 Cells

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Background: **The *in vitro* adaptive responses of δ opiate receptors (DOR) to chronic ethanol treatment have been well documented. The acute effects of ethanol on these receptors are not well characterized beyond its effect on ligand binding. The aim of this study was to evaluate the acute effects of clinically relevant concentrations of ethanol (50–200 mM) on the saturation binding kinetics, receptor/ligand internalization, and agonist stimulation of G-protein coupling in N18TG2 cells expressing the Flag epitope-tagged mouse DOR.**

Methods: **Confocal microscopy was used to localize Flag epitope-tagged DOR in N18TG2 cells. Saturation binding assays at 4°C and 37°C were conducted in the absence or presence of ethanol on cells not pretreated or pretreated with ethanol for 30 min at 37°C. Highly specific δ agonist, DPDPE ([D-Pen^2,D-Pen^5]enkephalin), was used in these studies. The effect of ethanol on agonist stimulation of G-protein coupling was examined using [^{35}S]GTPγS (guanosine-5′-O-(3-thio)triphosphate) binding to membranes. Agonist-mediated receptor internalization was examined using flow cytometry of cells labeled with the antiserum directed against the Flag epitope, and the ligand internalization was examined using [^{3}H]DPDPE.**

Results: **Ethanol decreased the binding of the agonist [^{3}H]DPDPE, and not the antagonist [^{3}H]diprenorphine, in a dose-dependent manner. These effects were temperature-dependent. Ethanol reversibly inhibited agonist stimulation of [^{35}S]GTPγS binding. In non-pretreated cells, ethanol decreased the rate of receptor/ligand internalization, but this effect was not seen in ethanol pretreated cells. Taken together, these results suggest that pretreatment of N18TG2 cells with ethanol induces compensatory mechanisms that allow the receptor to function efficiently in its presence.**

Conclusion: **Acute ethanol decreased the binding, agonist-mediated functional coupling and receptor/ligand internalization in N18TG2 cells expressing epitope-tagged DOR. In these cells, 30-min pretreatment with ethanol was sufficient to reverse these effects. (Key words: Desensitization; endocytosis; enkephalin; G-protein–coupled receptors; neuroblastoma.)**

ALCOHOLS have been extensively used in studies aimed at elucidating the mechanisms of anesthetic action. Ethanol produces progressive depression of the central nervous system and can induce general anesthesia at concentrations greater than 50 mM.[1] A number of biochemical studies have shown that ethanol also alters the processing, release, and receptor-binding properties of endogenous opioid peptides.[2–3] Among the different subtypes of opioid receptors, the δ is more susceptible to the acute and chronic effects of ethanol[4–7] than the μ or κ subtypes.

A number of aspects of receptor regulation, such as agonist-mediated endocytosis and receptor recycling, can best be studied using cultured cells expressing the δ opioid receptor (DOR). The binding of an agonist to the opioid receptor activates associated G proteins followed by induction of a number of second messenger systems. This is followed by a rapid agonist-induced internalization of the receptor, leading to uncoupling of receptors from G proteins and ultimately termination of signaling.[8–11]

Studies using neuronal cell lines expressing DOR have shown that chronic exposure to clinically relevant concentrations of ethanol (25–100 mM) leads to upregulation of DOR mRNA levels.[12] Acute exposure of cells to ethanol reversibly decreased the binding of δ-selective peptide [^{3}H]DADLE ([D-Ala^2-D-Leu^5]enkephalin).[13] However, little is known about the effects of acute ethanol on the immediate events that follow ligand binding to DOR, such as on the agonist stimulation of guanosine-5′-O-(3-thio)triphosphate ([^{35}S]GTPγS) binding to G proteins and agonist-mediated receptor/ligand internalization.

In this study, using transfected N18TG2 mouse neuro-

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blastoma cells with the Flag-tagged DOR, we studied the effects of acute ethanol treatment on DOR.

Materials and Methods

Transfection and Characterization of the Neuroblastoma Cells Expressing Epitope-tagged DORs

Cell Culture. Flag epitope (ADDDDKYD)-tagged δ opiate receptor was subcloned into pCDNA3 expression vector as previously described.[14] N18TG2 cells were transfected with 5 mg Qiagen (Qiagen Inc., Valencia, CA)-purified plasmid DNA using Lipofectin reagent (Life Technologies, Grand Island, NY). Colonies with stable expression were selected in a medium containing 500 μg/ml of geneticin. Colonies were tested for receptor expression by a binding assay using [^{3}H]diprenorphine.[14] Cells expressing the receptor (500,000 receptors/cell) were grown to confluence under 5% CO_2 in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 500 μg/ml geneticin. Cells were subcultured at a ratio of 1:5 with partial replacement of the media on the day before subculturing or collected at day 5 or 6.

Cell Staining and Immunofluorescence Microscopy. N18TG2 cells stably transfected with DOR were grown on coverslips and were treated without or with 100 nM agonist for 30 min or 24 h. After incubation, the cells were washed with ice-cold 20 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 1 mM $CaCl_2$ (TBS), fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). Fixed cells were washed with TBS, permeabilized and blocked with 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in Blotto (Pierce, Rockford, IL) (3% nonfat dry milk in 50 mM Tris-Cl, pH 7.5). Cells were incubated for 1 h at room temperature with 10 μg/ml primary antibody (anti-FLAG M1 diluted in Blotto), washed with TBS, incubated for 30 min with 2 μg/ml fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G (diluted in Blotto), washed with TBS and mounted on glass slides using Permount (Fisher Scientific, Pittsburgh, PA). Cells were examined using an oil-immersion objective and standard fluorescein epifluorescence optics, and confocal fluorescence microscopy was performed using a laser scanning microscope.

Binding Assays. Cells were plated on 24-well plates. After 24 h, the media was removed, and they were incubated with [^{3}H][D-Pen^2,D-Pen^5]enkephalin ([^{3}H]DPDPE; 2 nM final concentration) or [^{3}H]diprenorphine (0.9 nM final concentration) in Kreb's-Ringer's-HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer, pH 7.4 (buffer A) in a final volume of 300 μl. The incubations were kept for 30 min at 37°C. Nonspecific binding was determined in the presence of 100 nM DPDPE or diprenorphine and was 5–10% of the total binding. At the end of the incubation period, the plates were kept on ice, and wells were washed five times with 0.5 ml 50 mM Tris-Cl, pH 7.5. Cells were dissolved in 100 μl 1 N NaOH, collected, and neutralized with 100 μl 1 N HCl, and radioactivity was measured in Biosafe scintillation fluid (Beckman Coulter Inc., Fullerton, CA).

Membrane Preparation for [^{35}S]GTPγS Binding. Cells were washed twice with ice-cold PBS, pelleted at 800*g* for 3 min, and resuspended with 10 volumes of 5 mM Tris-Cl, pH 7.4, with protease inhibitors (10 μM leupeptin, 10 μM aprotinin, 1 μM pepstatin, 100 μg/ml bacitracin, 1 μM E64, 1 mM EDTA, 1 mM EGTA, 10 μM iodoacetamide). Cells were probe sonicated twice for 10 s using a Branson Sonifier cell disrupter (Branson Ultrasonic Corp., Danbury, CT) at setting 6 (chilling between sonications on ice for 30–60 s) followed by centrifugation at 5,000*g* for 15 min. The supernatant was centrifuged at 40,000*g* for 20 min at 4°C. The resulting pellet was diluted to 20 ml with 50 mM Tris-Cl and recentrifuged at 40,000*g*. The final pellet was resuspended in 50 mM Tris-Cl, pH 7.4, and frozen at −80°C in 100-μl aliquots (1–2 mg/ml). Protein estimation was with BCA protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Effect of Acute Ethanol on DOR in N18TG2 Cells

Effect of Ethanol on Ligand Binding to DOR. Two ligands, [^{3}H]DPDPE (a δ opioid-selective ligand that binds to the receptors present on the cell surface) and [^{3}H]diprenorphine (nonspecific antagonist that binds to both the cell surface as well as intracellular receptors) were used to examine the effect of ethanol (50–200 mM) on ligand binding. The concentrations of ethanol selected are within the clinically relevant range after *in vivo* administration (25–100 mM) and the reported IC_{50} of ethanol for [^{3}H]DADLE binding (200 mM) to brain membranes.

Effect of Ethanol on the Saturation Binding of [^{3}H]DPDPE to DOR. These experiments were conducted at either 37°C (agonist-mediated receptor internalization occurs) or at 4°C (agonist-mediated receptor internalization inhibited). A representation of the experiments performed is shown in table 1. Concentrations of [^{3}H]DPDPE from 0.1 to 20 nM (final concentration) were

Table 1. Experimental Groups

Group	Ethanol (mM)	Pretreatment with Ethanol at 37°C (min)	Temperature (°C)	Comments
Saturation binding of [^{3}H]DPDPE				
Control	—	—	4	No internalization
	—	—	37	Internalization
Test	50	0	4/37	Nonpretreated cells
	200	0	4/37	Nonpretreated cells
	50	30	4/37	Pretreated cells
	200	30	4/37	Pretreated cells
Agonist stimulation of [^{35}S]GTPγS binding				
Control	—	—	30	
Test	50	0	30	Nonpretreated cells
	200	0	30	Nonpretreated cells
	50	30	30	Pretreated cells
	200	30	30	Pretreated cells
Receptor/ligand internalization				
Control	—	—	37	
Test	50	0	37	Nonpretreated cells
	200	0	37	Nonpretreated cells
	50	30	37	Pretreated cells
	200	30	37	Pretreated cells

used in these studies. Nonspecific binding was determined in the presence of unlabeled DPDPE and was 5–10% of the total binding. Binding assays at 4°C were conducted for 16 h for equilibrium to be reached.

Effect of Ethanol on Agonist Stimulated [^{35}S]GTPγS Binding. Membranes (10 μg/tube) were mixed with various doses of DPDPE and preincubated for 10 min at 30°C. This was followed by the addition of assay buffer to yield a final concentration in 100 μl of 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiotreitol (added fresh), 50 μM guanosine-5′-diphosphate, and 50 pM [^{35}S]GTPγS. Tubes were incubated for 30 min at 30°C, and the reaction was terminated by diluting the sample with 2 ml ice-cold 50 mM Tris-Cl, pH 7.4, containing 5 mM $MgCl_2$ and 100 mM NaCl, and rapidly filtering the contents through glass fiber filters (no. 32, Schleicher and Schuell, Keene, NH). The filters were then washed three times with 2 ml buffer. Filters were placed in vials containing 400 μl of ethanol and 4 ml of Biosafe scintillation cocktail. Basal activity was defined as the difference between the [^{35}S]GTPγS binding in the absence and presence of 50 μM unlabeled GTPγS. To determine the increase in the [^{35}S]GTPγS binding over basal, the basal binding was subtracted for each dose of DPDPE, and the value was divided by the basal value and then multiplied by 100.[15,16] The experiment was performed in triplicate in the absence (control) or presence of ethanol (50/200 mM) or with membranes pretreated with buffer A/ethanol (50/200 mM) for 30 min at 37°C followed by centrifugation at 40,000*g* for 20 min to remove the ethanol. The [^{35}S]GTPγS assay was then conducted in the absence of ethanol.

Effect of Ethanol on Agonist-induced DOR Internalization. These studies were conducted using flow cytometry as previously described.[17] Briefly, 1–2 × 10^5 N18TG2 cells expressing Flag-tagged DOR were plated onto a 24-well plate. After 24 h, the cells (non-pretreated and pretreated with 50/200 mM ethanol) were incubated with 100 nM DPDPE in buffer A. At the end of the incubation, cells were chilled to 4°C, washed three times with 0.5 ml PBS, and incubated with 10 μg/ml primary antiserum (anti-Flag M1 antiserum) in 50% fetal bovine serum in PBS for 1 h. Cells were washed with 1% fetal bovine serum in PBS and incubated with 5 μg/ml fluorescein isothiocynate–conjugated goat antimouse immunoglobulin G. Cells were washed with 1% fetal bovine serum in PBS followed by a PBS wash, collected from the wells with 5 mM EDTA, and analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Inc., San Jose, CA). Live cells were gated by light scatter or exclusion of propidium iodide, and 5,000–10,000 cells were acquired for each time point. The mean fluorescence of all live cells in the absence of DPDPE minus mean fluorescence of cells stained only with fluorescein isothiocyanate–conjugated second antibody was taken as total surface receptor expressed by the cells and used for calculation of the percentage of receptor internalized by treatment with DPDPE.[18]

Effect of Ethanol on [^{3}H]DPDPE Internalization. N18TG2 cells expressing the Flag-tagged DOR (1–2 ×

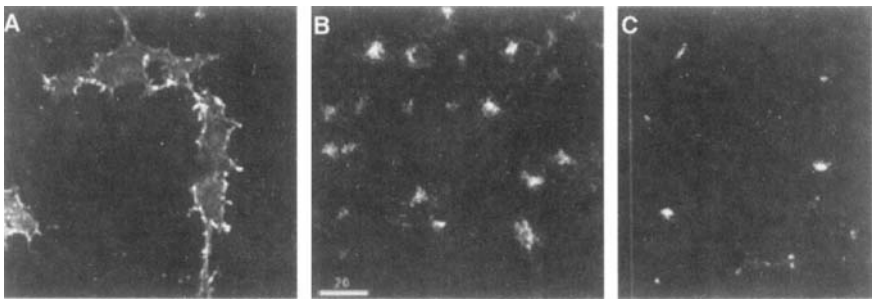

Fig. 1. Neuroblastoma cells expressing δ opioid receptors. Cells were incubated in the absence (*A*) or presence of 100 nM DADLE for 30 min (*B*) or 24 h (*C*). Fixation, permeabilization, staining, and confocal microscopy of the receptors with the anti-Flag antibody were conducted as described in Materials and Methods. Bright staining of the plasma membrane is seen in (*A*), whereas prominent intracellular staining is seen inside the cells in (*B*) and (*C*).

10^5 cells) were plated into 24-well plates. After 24 h, the medium was removed, and cells (non-pretreated/pretreated with ethanol 50/200 mM) were incubated with [^{3}H]DPDPE (2 nM final concentration) in a final volume of 300 μl. At the end of the incubation period, the plates were chilled at 4°C, and cells washed in 50 mM Tris-Cl, pH 7.5, were collected to obtain the total binding. The amount of ligand internalized was determined by washing another set of wells with ice-cold 0.2 M sodium acetate buffer, pH 4.8, containing 500 mM sodium chloride, the acid buffer that has been previously shown to remove cell surface binding.[19] The cells were washed with 50 mM Tris-Cl, pH 7.5, dissolved in 1 N NaOH, neutralized with 1 N HCl, and radioactivity was measured in Biosafe scintillation fluid.

Statistical Analysis

Saturation binding data for [^{3}H]DPDPE was analyzed using GraphPad Prism software (San Diego, CA). Dunnett's test was used for the statistical analysis between control and ethanol (50/200 mM final concentration) in the experiments involving receptor/ligand internalization and [^{35}S]GTPγS binding.

Materials

Lipofectin and geneticin (G418) were purchased from Life Technologies Inc. (Grand Island, NY); [^{3}H]diprenorphine, [^{3}H]DPDPE, and [^{35}S]GTPγS were from DuPont NEN (Boston, MA); anti-FLAG M1 antibody was from Sigma (St. Louis, MO); fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G was from Vector Laboratories (Burlingame, CA); diprenorphine and DPDPE were from Peninsula Laboratories (Merseyside, United Kingdom); Biosafe Scintillation fluid was from Beckman; BCA protein assay reagent was from Pierce (Rockford, IL); and glass fiber filters (no.32) were from Schleicher and Schuell (Keene, NH). All other reagents were of analytical grade and purchased from Sigma.

Results

Transfection and Characterization of the Neuroblastoma Cells Expressing Epitope-tagged DORs

Full-length, Flag-tagged, wild-type mouse DOR cDNA was stably transfected into N18TG2 mouse neuroblastoma cells, and 48 individual colonies were isolated; six different colonies were expanded and further studied. The cell lines were characterized in terms of their binding affinity for DPDPE. The receptors expressed in N18TG2 cells exhibited a high affinity for DPDPE (K_d 4.1 nM). The distribution of DORs in N18TG2 cells was examined by confocal fluorescence microscopy after acute (30 min) and chronic (24 h) exposure to 100 nM DPDPE in the incubation media (fig. 1). In the absence of the agonist, receptors were mainly located on the plasma membrane (fig. 1A); acute exposure to the agonist resulted in the displacement of the receptors from the cell surface to the cytoplasmic side (fig. 1B), whereas 24-h exposure resulted in a significant reduction of receptor fluorescence (fig. 1C). These results showed that acute and chronic exposure to DPDPE differentially altered the location and degree of receptor-associated fluorescence in N18TG2 cells.

Effect of Ethanol on Binding of Ligand to Receptor

This set of experiments was aimed at determining the effect of ethanol on the binding of [^{3}H]DPDPE (a DOR-selective ligand that binds to the cell surface receptors) and [^{3}H]diprenorphine (a nonspecific opioid receptor antagonist that binds to both cell surface as well as intracellular receptors) to Flag-tagged DOR in N18TG2 cells. Increasing doses of ethanol did not significantly affect [^{3}H]diprenorphine binding, indicating that the total number of receptors remained constant for the time

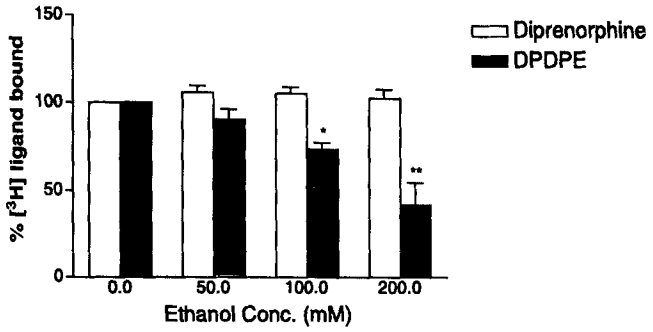

Fig. 2. Effect of ethanol on ligand binding to epitope-tagged N18TG2 δ opioid receptor. Cells were incubated with [^{3}H]diprenorphine or [^{3}H]DPDPE as described in Materials and Methods. Specific binding in the absence of ethanol was taken as 100%. Results are the mean ± SD of three independent experiments; values that represent significant differences from control values (in the absence of ethanol) are indicated. *P < 0.05; **P < 0.01 (Dunnett test).

period used during the assay (fig. 2). However, ethanol decreased [^{3}H]DPDPE binding to the receptors (fig. 2), suggesting that it caused either a change in the affinity of the receptor for this ligand, increased the dissociation of the ligand from the receptor, or affected the rates of receptor internalization.

Effect of Ethanol on the Saturation Binding of [^{3}H]DPDPE to DOR

Saturation binding data for [^{3}H]DPDPE were fit by nonlinear analysis of one or two site-binding models using the Graph Prism software. The data were best fit by a single saturable binding site (P < 0.5). Scatchard analysis of the data showed that, under conditions in which receptor internalization is prevented (4°C), the K_d of [^{3}H]DPDPE for DOR was 4.1 ± 0.3 nM in control cells. In non-pretreated cells (fig. 3A), ethanol increased the K_d, *i.e.*, decreased the affinity, of [^{3}H]DPDPE by approximately 1.4-fold at 50 mM and by threefold at 200 mM (table 2). In pretreated cells (fig. 3B), ethanol increased the K_d by 1.1-fold at 50 mM and by 2.2-fold at 200 mM (table 2).

When the saturation binding assays were conducted at 37°C (conditions under which agonist-mediated receptor internalization occurs) for 30 min, we found that in non-pretreated cells, ethanol increased the K_d of [^{3}H]DPDPE binding by 2.3-fold at 50 mM and by 19.6-fold at 200 mM (table 2) over control values. However, in pretreated cells, ethanol increased the K_d by 2.1-fold at 50 mM and only by 3.1-fold at 200 mM in pretreated cells. These results suggest that the magnitude of the effect of

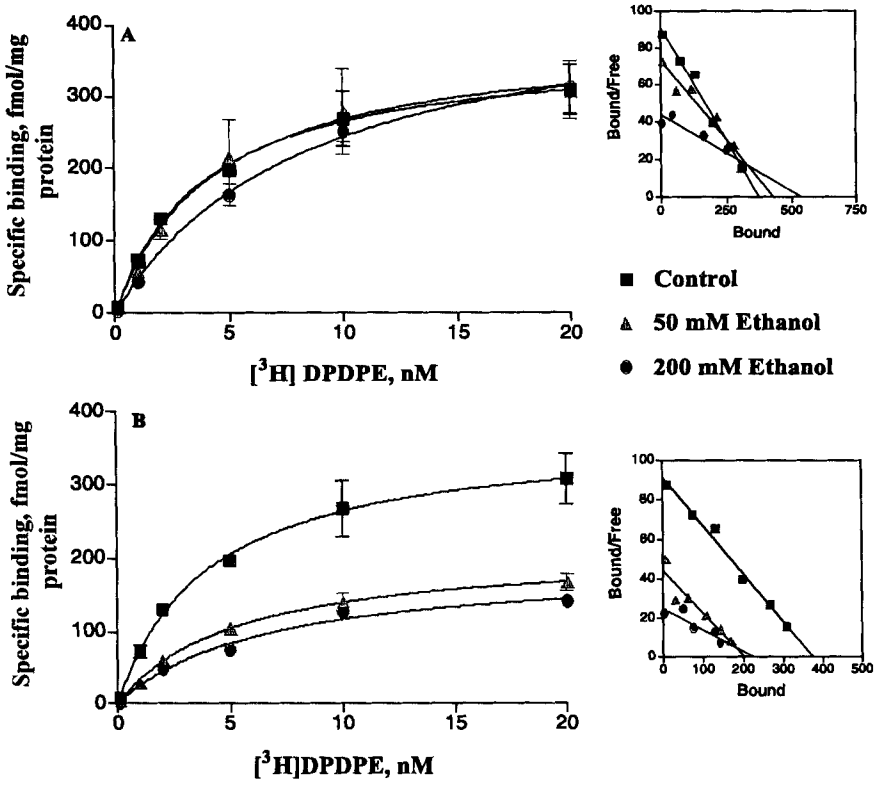

Fig. 3. Effect of ethanol on [^{3}H]DPDPE saturation binding to δ opioid receptors expressed in N18TG2 cells. (*A*) Cells were pretreated with buffer alone for 30 min at 37°C followed by incubation with [^{3}H]DPDPE in the absence (control) or presence of ethanol at 4°C for 16 h. Scatchard analysis of the data is shown (*upper right*). (*B*) Cells were pretreated with ethanol for 30 min at 37°C followed by incubation with [^{3}H]DPDPE in the presence of ethanol at 4°C for 16 h. Cells not exposed to ethanol throughout the assay were taken as control. Scatchard analysis of the data is shown (*lower right*). Results are the mean ± SD of three independent experiments.

Table 2. Scatchard Analysis of Effect of Ethanol on [^{3}H]DPDPE Binding

Group	K_d (nM)		B_{max} (fmol/mg protein)	
	4°C	37°C	4°C	37°C
Control	4.1 ± 0.30	5.7 ± 0.7	373.7 ± 9.63	2784 ± 126
Nonpretreated cells				
50 mM ethanol	5.9 ± 0.66	13.2 ± 0.92*	430.8 ± 20.44	2910 ± 108
200 mM ethanol	12.2 ± 0.82†	111.4 ± 8.65†	537.1 ± 18.56†	7424 ± 652†
Pretreated cells				
50 mM ethanol	4.6 ± 0.37	12.0 ± 0.87*	203.0 ± 5.79†	3143 ± 119
200 mM ethanol	9.0 ± 1.74†	17.5 ± 1.10†	225.0 ± 20.46†	3160 ± 113

Binding of [^{3}H]DPDPE to N18TG2 cells plated in 24-well plates was determined as described in Materials and Methods. Incubations were carried out either for 30 min at 37°C or for 16 h at 4°C. Cells that were not subject to ethanol treatment are designated as control. Results are the mean ± SD of duplicate determinations from three independent experiments; values that represent significant differences from respective controls are indicated.

* $P < 0.05$.

† $P < 0.01$ (Dunnett test).

ethanol on the affinity of the receptor for the ligand is affected by temperature. Comparison of the effects of ethanol in non-pretreated and pretreated cells shows that there are significant differences in K_d at 200 mM ethanol concentration in binding assays conducted at 4°C/37°C ($P < 0.01$).

Effect of Ethanol on Agonist-stimulated [^{35}S]GTPγS Binding

To examine if ethanol affected functional coupling of receptor to G proteins, [^{35}S]GTPγS binding to membranes prepared from N18TG2 cells transfected with DOR were conducted. In control cells (not exposed to ethanol), we found an increase in [^{35}S]GTPγS binding in response to increasing concentrations of DPDPE (fig. 4A). The presence of ethanol caused a significant reduction in the agonist-stimulated increases in [^{35}S]GTPγS binding to membranes. In contrast, when the membranes were pretreated with ethanol, centrifuged to remove the alcohol, and the [^{35}S]GTPγS binding was conducted in the absence of ethanol, no differences were seen between control and ethanol pretreatment (fig. 4B).

Effect of Ethanol on Agonist-induced Receptor Internalization

Our results show that exposure to the agonist caused a rapid and robust increase in the internalization of DOR in control cells (fig. 5). In the presence of ethanol, agonist-mediated receptor internalization was significantly reduced; only approximately 10–20% receptor internalized in the initial 10 min (fig. 5A). Interestingly, cells pretreated with ethanol for 30 min at 37°C did not show any difference in the kinetics of agonist-mediated receptor internalization when compared with control cells (fig. 5B).

Effect of Ethanol on [^{3}H]DPDPE Internalization

To examine the effect of ethanol on the time course of ligand internalized as part of the receptor-ligand complex, cells were incubated with [^{3}H]DPDPE (2 nM) in the absence and presence of ethanol. Figure 6A shows that in control cells, there was an increase in ligand internalization with time. This was similar to that observed for DOR internalization (fig. 5A) but lesser in magnitude because the concentration of [^{3}H]DPDPE used here is 2 nM. As in the case of receptor internalization, ethanol decreased ligand internalization to a significant extent. In cells pretreated with alcohol for 30 min, no differences were seen in the kinetics of [^{3}H]DPDPE internalization between control and pretreated cells (fig. 6B).

Discussion

Alcohols have been useful in providing an insight into the probable mechanisms of action of anesthetics because they can produce an anesthetic state. Ethanol, when administered at concentrations greater than 50 mM, induces general anesthesia.[1] Ethanol modulates the opioid system by modifying the processing, release, and receptor-binding characteristics of endogenous ligands,[2,3] and changes in the endogenous opioid system could contribute to ethanol intoxication and adaptive responses.[20] In this study, we stably transfected N18TG2 cells with Flag-tagged DOR. We found that receptors

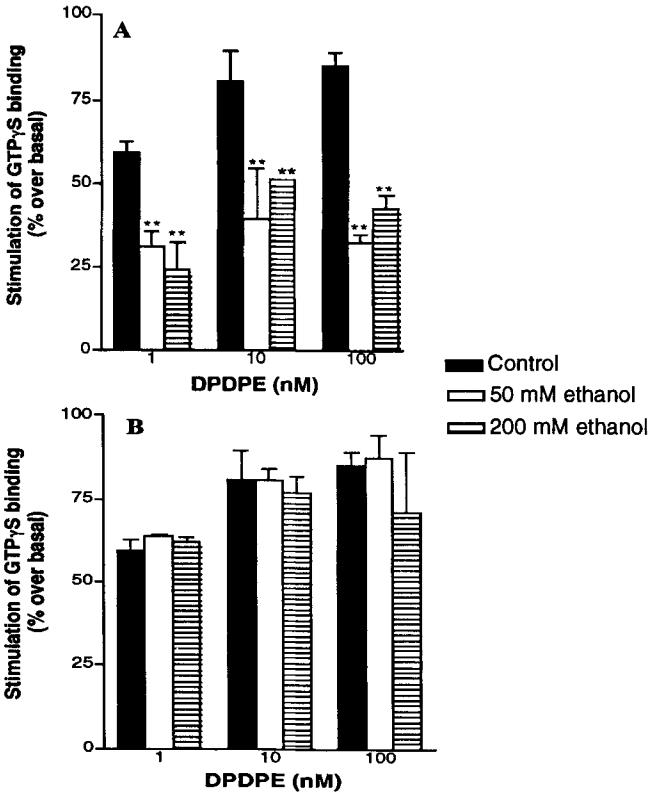

Fig. 4. Effect of ethanol on agonist-stimulated [^{35}S]GTPγS binding in N18TG2 membranes. Membranes were prepared and pretreated with buffer alone (*A*) or ethanol (*B*) for 30 min at 37°C. The increase in [^{35}S]GTPγS on agonist stimulation was determined as described in Materials and Methods. Results are the mean ± SD of triplicate determinations from two independent experiments; values that represent significant differences from control values (in the absence of ethanol) are indicated. **$P < 0.01$ (Dunnett test).

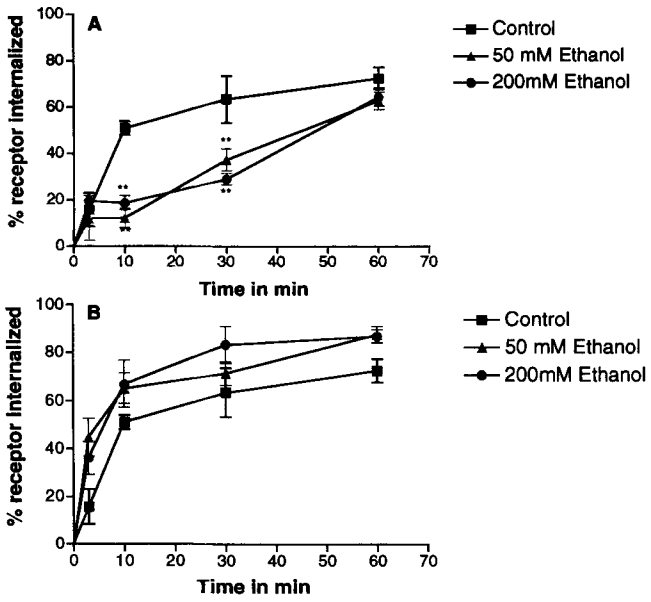

Fig. 5. Effect of ethanol on the internalization of δ opioid receptors. Cells were pretreated with buffer alone (*A*) or ethanol (*B*) for 30 min at 37°C. The kinetics of internalization of the epitope-tagged receptor on incubation with 100 nM DPDPE was conducted by flow cytometry as described in Materials and Methods. Results are the mean ± SD of triplicate determinations from three independent experiments; values that represent significant differences from control values (in the absence of ethanol) are indicated. **$P < 0.01$ (Dunnett test).

expressed in N18TG2 cells exhibit a high affinity for DPDPE (K_d 4.1 nM) similar to the reported affinity for DOR.[17,21] Confocal fluorescence microscopy showed that, in the absence of the agonist, receptors were mainly located on the plasma membrane; acute exposure to the agonist resulted in the displacement of the receptors from the cell surface to the cytoplasm, whereas during chronic exposure there is a significant reduction of receptor fluorescence, suggesting that receptor degradation is taking place. This behavior is similar to that of endogenous DOR in NG108-15 cells[21] and the receptors expressed in CHO cells.[17]

Acute exposure of N18TG2 cells to ethanol inhibited the binding of δ-selective agonist, [^{3}H]DPDPE, in a dose-dependent manner, but not the binding on the antagonist, [^{3}H]diprenorphine. Ethanol decreased the affinity of the receptor for [^{3}H]DPDPE. These effects were more pronounced at 37°C than at 4°C. Hiller *et al.*,[4] using rat brain membranes, were the first to demonstrate that ethanol selectively inhibited the binding of enkephalins to the DOR in a dose-dependent manner. They showed that the inhibition was reversible and that the potency increased with the chain length of n-alcohols. Furthermore, the inhibition of enkephalin binding was found to be caused by a decrease in the affinity of the receptor for the ligand that was caused by an increase in the rate of dissociation of the ligand-receptor complex. Increasing the temperature of incubation exacerbated the inhibitory effects of alcohols.[13] Charness *et al.*[22] used the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 to show that acute treatment with ethanol caused an inhibition of ligand binding, whereas chronic treatment caused an increase in binding due to an increase in the maximum number of binding sites (B_{max}). We found that acute treatment of N18TG2 expressing DOR with 200 mM ethanol causes a substantial change in affinity and a significant increase in the B_{max} (table 2) at 37°C. This could be due to the fact that our studies were conducted in attached cells, whereas most studies on the acute effect of ethanol on DORs were conducted either

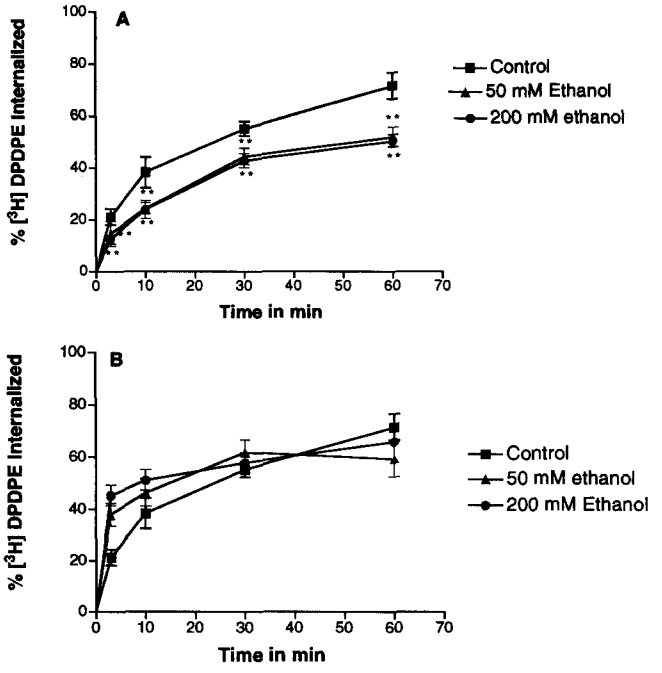

Fig. 6. Effect of ethanol on [^{3}H]DPDPE internalization in N18TG2 cells expressing δ opioid receptors. Cells were pretreated with buffer alone (*A*) or ethanol (*B*) for 30 min at 37°C. The kinetics of internalization of [^{3}H]DPDPE was conducted as described in Materials and Methods. Results are the mean ± SD of triplicate determinations from three independent experiments; values that represent significant differences from control values (in the absence of ethanol) are indicated. **$P < 0.01$ (Dunnett test).

in membrane preparations or in cells in suspension. The reported change in B_{max} observed with 200 mM ethanol may not reflect a real value because the binding curve did not exhibit saturation even at the highest concentration of [^{3}H]DPDPE (20 nM) used in the assay.

Several studies have implicated G proteins as one of the sites of action of ethanol.[23] In this study, we show that acute treatment with ethanol reversibly inhibits agonist stimulation of [^{35}S]GTPγS binding. In NG108-15 cells, chronic treatment with ethanol causes a significant reduction in the level of $G_{s\alpha}$ and no significant changes in $G_{i\alpha}$. In N1E-15 cells, chronic treatment with ethanol causes a dose-dependent increase in the levels of $G_{i\alpha}$ and a time-dependent decrease in the levels of G_{sa}. In contrast, in N18TG2 cells, there is no significant change in the levels of $G_{i\alpha}$ or G_{sa}, suggesting that these cells are more resistant to the chronic effects of ethanol.[24] Thus, it seems that ethanol has a short-term effect on functional coupling to opioid receptors as well as long-term effects on the levels of G proteins. Studies have also shown that acute exposure of cells to ethanol leads to an inhibition of a nucleoside transporter, resulting in an increase in intracellular adenosine, activation of adenosine A2 receptors, and an increase in intracellular cyclic adenosine monophosphate levels that, in turn, affected the basal levels of DOR.[25,26] However, it is not known if this process also occurs in closely related cell lines such as N18TG2, N4TG1, N1E-115, and the C1300 neuroblastomas.

The number of receptors present on the cell surface is affected by its internalization and recycling. It has been shown that the binding of a number of peptide ligands to their endogenous G-protein-coupled receptors is followed by the internalization of the receptor-ligand complex both in neuronal cells as well as in host cells containing recombinant receptor.[8-10] It has been postulated that this process is important for receptor desensitization and/or resensitization.[27-29] Significant internalization occurs within 10 min, and recycling of receptor has been demonstrated after removal of agonist.[30,31] In this study, we show that in control cells, exposure to the agonist causes a rapid and robust internalization with a half-time of 10 min. These kinetics of receptor internalization are similar to that previously reported for DOR expressed in CHO or HEK-293 cells.[32,33] Ethanol decreases the rate of ligand internalization as well as that of receptor internalization at 37°C. This could be due to a direct effect of ethanol on membrane-associated phospholipase D. This enzyme hydrolysis phosphatidylcholine to generate phosphatidic acid, which has been implicated in vesicular trafficking.[34,35] However, in the presence of n-alcohols, phospholipase D catalyzes a transphosphatidylation reaction generating phosphatidylalcohol at the expense of phosphatidic acid.[36] The decrease in phosphatidic acid levels caused by ethanol could account for the decreased rates of receptor, and ligand internalization till levels were restored by the action of other enzymes capable of generating phosphatidic acid.

We found that in cells pretreated with ethanol for 30 min at 37°C, the rates of ligand and receptor internalization are similar between control and ethanol-treated cells. This suggests that N18TG2 cells adjust quickly to the effects of ethanol either by limiting its entry into the cell or by compensating for its effects within the plasma membrane. Charness *et al.*[12] have shown that ethanol differentially modulates signal transduction proteins in N18TG2, N4TG1, N1E-115, and NG108-15 neuronal cell lines. These cell lines are derived from a common ancestor C1300 neuroblastoma but show different sensitivities to chronic ethanol treatment. The effects of ethanol vary in different brain regions and among different individu-

als; therefore, the understanding of the differential effects of ethanol in these cell lines may provide useful insights into the heritable component of alcoholism. In addition, animal studies have shown that tolerance to ethanol may occur after as little as 30 min of ethanol administration.[37] We are performing further analysis of these related cell lines to identify the factors that endow some but not all cells of neuronal origin with the capacity to adapt to the effects of ethanol and other anesthetics.

In conclusion, this study shows that acute ethanol treatment affects the binding, agonist-mediated functional coupling to G proteins as well as receptor/ligand internalization in N18TG2 cells expressing Flag epitope-tagged DORs. In addition, 30-min pretreatment with ethanol induces compensatory mechanisms in these cells, which allow the receptor to function efficiently in its presence.

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