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G_i Is Involved in Ethanol Inhibition of L-Type Calcium Channels in Undifferentiated but Not Differentiated PC-12 Cells

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SUMMARY

The effects of acute exposure to 25 mM ethanol on high voltage-activated, L-type Ca^{2+} channels in undifferentiated and nerve growth factor-treated pheochromocytoma (PC-12) cells were examined using conventional, whole-cell, patch-clamp techniques. Acute exposure to 25 mM ethanol inhibited macroscopic L-type Ca^{2+} currents in undifferentiated PC-12 cells significantly more than in nerve growth factor-treated PC-12 cells. Intracellular infusion with guanosine-5′-*O*-(2-thio)diphosphate or pretreatment with pertussis toxin reduced ethanol inhibition in undifferentiated cells without altering inhibition in nerve growth factor-treated cells, suggesting the involvement of a G protein in ethanol inhibition of Ca^{2+} channels in undifferentiated cells. Intracellular infusion with an affinity-purified antibody that recognizes the carboxyl termini of α_{i1} and α_{i2} significantly reduced ethanol inhibition in undifferentiated cells, in contrast to the effects of antibodies that recognize the carboxyl termini of α_{oA} and α_{oB}. None of these antibodies reduced ethanol inhibition in nerve growth factor-treated cells. These results indicate that $G_{i1\alpha}$ or $G_{i2\alpha}$ mediates ethanol inhibition of L-type Ca^{2+} channel currents in undifferentiated but not in nerve growth factor-treated PC-12 cells.

DHP-sensitive, L-type Ca^{2+} channels have been shown to be inhibited by ethanol in UND (1–4) and NGF-treated PC-12 cells (3, 4), rat neurohypophysial terminals (5, 6), differentiated N1E-115 and NG108–15 cells (7), and rat pinealocytes (8). In all cases tested, acute exposure to ethanol causes a significant reduction in Ca^{2+} current amplitude, but the mechanism that underlies the modulation of Ca^{2+} currents by ethanol is, as yet, unknown.

We previously showed that DHP-sensitive, L-type Ca^{2+} channels in UND PC-12 cells are more sensitive to the effects of an acute exposure to ethanol than are DHP-sensitive, L-type channels in NGF-treated cells (4). The reduction in current produced by ethanol was concentration dependent and was not due to a shift in the current-voltage relationship of the channels. The difference in ethanol inhibition of Ca^{2+} currents in the two cell types was not due to the expression of conotoxin-sensitive, N-type channels in NGF-treated cells, because conotoxin did not alter the level of ethanol inhibition in these cells. In both cell types, the effect of ethanol was greatest when currents were evoked from a depolarized holding potential, suggesting that ethanol has a greater effect on the inactivated state than on the resting or activated state of the channel. Acute ethanol treatment produced a hyperpolarizing shift in steady state inactivation of Ca^{2+} channels that could account for approximately one half of the ethanol inhibition in UND cells and virtually all of the ethanol inhibition in NGF-treated cells (4). In this paper, we explore the basis for the additional inhibition observed in UND cells.

There is growing evidence that neurotransmitter inhibition of voltage-dependent Ca^{2+} channels is mediated by the PTX-sensitive G_o family of G proteins, the most abundant G proteins in the nervous system (for review, see Refs. 9 and 10). G_o proteins are usually more potent than G_i, the other family of PTX-sensitive G proteins, in reconstituting neurotransmitter inhibition of Ca^{2+} channels (10–12). In the case of bradykinin-induced inhibition, however, α_o, α_{i1}, and α_{i2} were equally effective in restoring channel inhibition after PTX pretreatment (11). In most cases, the Ca^{2+} channel type affected is the high voltage-activated, Ω-conotoxin GVIA-sensitive, N-type channel (12, 13). There is one report that low voltage-activated T-type channels are also inhibited (11). One report indicates that two splice variants of $G_{o\alpha}$, namely $G_{o1\alpha}$ ($G_{oA\alpha}$) and $G_{o2\alpha}$ ($G_{oB\alpha}$), mediate muscarinic M_4 receptor-

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ABBREVIATIONS: DHP, dihydropyridine; UND, undifferentiated; NGF, nerve growth factor; PTX, pertussis toxin; DADLE, D-Ala-D-Leu-enkephalinamide; $V_{½}$, half-maximal inactivation potential; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; TBS-T, Tris-buffered saline/Tween.

and somatostatin receptor-induced inhibition of L-type Ca^{2+} currents in a clonal rat pituitary cell line (14).

PC-12 cells express α subunits of several PTX-sensitive G proteins, i.e., α_{i1}, α_{i2}, α_{i3}, and α_o, which exists in two alternatively spliced forms, α_{oA} and α_{oB} (15–19). In UND cells, the levels of both α_{i1} protein (16, 19) and mRNA (18) appear to be less than the levels of α_{i2} protein and mRNA. After differentiation with NGF, there is an increase in both protein and mRNA levels of α_o, in particular the α_{oA} form (15, 19), whereas levels of both α_{i1} and α_{i2} are increased 2–2.5-fold after 2–4 days of NGF treatment and return to levels observed in UND cells after 4–7 days of treatment.

In the present study, we demonstrate that the PTX-sensitive G protein G_i is involved in ethanol inhibition of currents in UND cells but not in NGF-treated cells. We propose that the difference in ethanol sensitivity of L-type Ca^{2+} channels in the two cell types, which we have shown previously (3, 4), is due to the involvement of this G protein in UND cells but not in NGF-treated cells. To the best of our knowledge, this is the first time that G_i has been shown to be involved in inhibition of DHP-sensitive, L-type Ca^{2+} channel activity.

Materials and Methods

Cell cultures. PC-12 cells obtained from Dr. E. Shooter (Stanford University, Stanford, CA) were grown in Dulbecco's modified Eagle's medium containing L-glutamine and buffered with 3.7 g/liter $NaHCO_3$ and 15 mM HEPES, in 60-mm Corning tissue culture dishes. The medium also contained 5% fetal calf serum (Sigma), 10% heat-inactivated horse serum (J.H.R. Biosciences), 50 units/ml penicillin G (Sigma), and 50 μg/ml streptomycin (Sigma). Cell cultures, retrieved from −80° storage every 2 months, were grown in a 10% CO_2 incubator at 37° and passaged every week, and the medium was changed every 2–3 days. Parallel cultures were left untreated or were treated with 50 ng/ml of the 2.5 S fraction of NGF (Harlan Bioproducts for Science) for 7–14 days. Processes were evident after 2 days of NGF treatment. Both cell types were triturated and replated on 12-mm, round, glass coverslips approximately 36 hr before recording. This procedure kept most NGF-induced neural-like processes to less than half the size of the cell body, thus improving the voltage clamp in these cells. When these cells were compared with NGF-treated cells with short processes, there was no difference in the currents or the ethanol inhibition of the currents.

PTX application. Both cell types were incubated with 200 ng/ml PTX (List Laboratories) at 37° for 24 hr. For control experiments, cells were similarly incubated for 24 hr with PTX that had been heat-inactivated by heating to 95° for 30 min.

Electrophysiological methods. After the cells were washed with 100 ml of a solution containing 10 mM $CaCl_2$, 10 mM HEPES-CsOH, pH 7.4, 1 mM $MgCl_2$, 125 mM tetraethylammonium chloride, and 19 mM glucose, macroscopic Ca^{2+} currents were obtained at room temperature (22–25°). Studies were carried out according to the method of Hamill *et al.* (20), using the whole-cell patch clamp technique with a Dagan 8900 patch-clamp amplifier. Electrodes were coated with Sylgard to reduce pipet capacitance and were fire polished to resistances of 1.5–2.5 MΩ before recording. Because of the high input resistance and small currents (average peak current amplitudes for UND cells, 36.0 ± 2.7 pA, n = 72; average peak current amplitudes for NGF-treated cells, 99.3 ± 9.3 pA, n = 53), we did not compensate for series resistance, which was 22.4 ± 0.5 and 19.2 ± 1.3 MΩ for UND and NGF-treated cells, respectively. Whole-cell patch pipet tips were filled with a solution containing 130 mM CsCl, 10 mM EGTA, 2 mM $MgCl_2$, 20 mM HEPES-CsOH, pH 7.2, 2 mM Mg-ATP, and 0.2 mM cAMP and were backfilled with the same solution to which either guanine nucleotide analogs (GTPγS and GDPβS, obtained from Calbiochem) or heat-inactivated or active antibodies directed toward specific G proteins were added. After the formation of a gigaseal, cells were lifted off the coverslip and superfused (rate, 50 μl/min) with a solution containing 20 mM $BaCl_2$, 10 mM HEPES-$BaOH_2$, pH 7.4, and 125 mM tetraethylammonium chloride, without or with 25 mM ethanol. Gentle mouth suction was used to rupture the patch of membrane under the electrode, allowing the contents of the patch electrode to dialyze against the contents of the interior of the cell for at least 2 min before control currents were recorded. Because Ca^{2+} currents can run down during the course of experiments using the conventional whole-cell recording technique (21), protocols were designed so that they were completed in <15 min, at which time ~90% of the current still remained in the absence of ethanol.

pClamp software (Axon Instruments) was used to collect and analyze records. A *P/N* protocol was used to subtract leakage and capacitative currents from all records (analysis done off-line). Currents were recorded from the same cells before exposure, during acute exposure to 25 mM ethanol, and after ethanol exposure. Peak current amplitude was determined by averaging all data points that were acquired between 20 and 80 msec of the 100-msec depolarization. Data are expressed as means ± standard errors, and a two-tailed Student's t test was used to determine the significance of differences from control values.

Peptide conjugation and immunization. For the production of the AI12 antiserum, peptide (sequence, KNNLKDCGLF) was coupled to the carrier protein keyhole limpet hemocyanin using glutaraldehyde, essentially as described by Goldsmith *et al.* (22). BC1 antiserum was generated with peptide [sequence, (C)SWDSFLKIWN] coupled by its cysteine residue, with *m*-maleimidobenzoyl-*n*-hydroxysuccinimide ester as the coupling reagent, as described by Green *et al.* (23). Antibodies were raised in female New Zealand White rabbits according to the procedure described by Green *et al.* (23).

Affinity purification of antibodies was accomplished by application of crude antiserum to a column of immobilized peptide. The affinity columns were prepared with Affi-gel 15 according to the procedure outlined by Bio-Rad. After extensive washing with 0.05 M glycine, pH 7.5, 0.5 M NaCl, bound antibodies were eluted with 0.1 M glycine, pH 2.5, 0.5 M NaCl (24). Eluted antibodies were immediately neutralized to pH 7.5, dialyzed and concentrated with Centriprep concentrators (M_r 30,000 cut-off; Amicon) at 3000 × g in phosphate-buffered saline, pH 7.5, and stored at −80°, in aliquots, at a concentration of 1 mg/ml. The affinity-purified AI12 antibody recognized the α subunits of G_{i1} and G_{i2} purified from bovine brain but did not recognize the β or γ subunits or the α subunits of G_{oA}, G_{oB}, or G_s (25). The affinity-purified BC1 antibody recognized only the β subunit of purified G proteins and only a 35/36-kDa doublet in bovine brain membranes. For electrophysiological studies, the AI12 antibody was freshly diluted at 1/7000 in the patch electrode solution, such that the final concentration was 142.8 ng/ml.

Affinity-purified antibodies directed against unique sequences near the carboxyl termini of $G_{oA\alpha}$ (GOA antibody) and $G_{oB\alpha}$ (GOB antibody) were generous gifts of Dr. Tomiko Asano (Institute for Developmental Research, Aichi, Japan) (17). GOA and GOB antibodies were diluted 1/15,000 (final concentration, 50 ng/ml) and 1/5000 (final concentration, 60 ng/ml), respectively, in the patch electrode solution before each experiment. For controls, all antibodies were heat-inactivated at 95° for 30 min and then diluted in the patch electrode solution to the same final concentration as the active antibodies.

Immunoblots. Crude cell membranes were prepared as described previously (19), with slight modifications. Briefly, the harvested cells were resuspended in 2 ml of buffer containing 50 mM Tris, pH 7.6, 6 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. Cells were lyzed by three cycles of freezing (in liquid N_2) and thawing and careful trituration with a 1-ml tuberculin syringe to minimize clumping. The cell lysate was then centrifuged at 10,000 rpm in an SS34 rotor at 4° for 25 min. The supernatant was decanted and the crude membrane

pellet was resuspended in 0.5 ml of ice-cold buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol, with a tuberculin syringe. The protein concentration was determined using the Bio-Rad assay, with immunoglobulin as the standard. Fifty micrograms of PC-12 cell membrane protein were applied to lanes of an 11% sodium dodecyl sulfate-polyacrylamide gel. Mixed G proteins (0.5 μg) purified from bovine brain were used as a positive control. The resolved proteins were transferred to nitrocellulose membranes as described by Towbin *et al.* (26). The membranes were blocked for 30 min at room temperature with 5% nonfat dried milk diluted in TBS-T (20 mM Tris, pH 7.4, 137 mM NaCl, 0.05% Tween 20), washed with TBS-T, and then incubated overnight at room temperature in the presence of affinity-purified antibodies diluted in TBS-T containing 0.5 M NaCl. Blots were extensively washed and then incubated for 1 hr at room temperature with a secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit IgG; Amersham) diluted in TBS-T. After extensive washing of the blots, specific bands were visualized by luminescence using enhanced chemiluminescence Western blot reagent (Amersham) and Kodak XAR film.

Results

Ethanol inhibition of Ca^{2+} currents. Fig. 1A shows the effect of a 5-min exposure to 25 mM ethanol on calcium currents evoked by a depolarizing voltage step from a holding potential of −40 mV. Shown are representative traces in which peak current amplitudes in an UND cell (Fig. 1A, *top*) and a NGF-treated cell (Fig. 1A, *bottom*) were reduced 41% and 21%, respectively, by ethanol. The reduction of current by ethanol in UND cells (average for group, 41.9 ± 5.3%; n = 11) was significantly ($p < 0.05$) greater than the reduction of current produced in NGF-treated cells (average for group, 20.6 ± 3.4; n = 8). The time course of rundown of Ca^{2+} currents (Fig. 1B) indicates that, at any given time after the whole-cell configuration is achieved in UND PC-12 cells, the barium current in the presence of 25 mM ethanol is less than the current recorded in the absence of ethanol. In both cell types, the inhibition produced by acute exposure to ethanol takes several minutes to reach maximum levels (4), suggesting that the effect of ethanol is not a simple interaction with the channel and may involve intracellular mechanisms or pathways. The reduction of current was reversible in both cell types but typically required several minutes to return to 90 ± 3.9% (n = 28) of current values in the absence of ethanol in UND cells and 92.3 ± 3.7% (n = 14) of current values in the absence of ethanol in NGF-treated cells.

Involvement of G proteins in ethanol inhibition of currents. To determine whether Ca^{2+} currents in our PC-12 cells were affected by G proteins, we used the nonhydrolyzable GTP analog GTPγS. This compound irreversibly activates G proteins. Activation by GTPγS has been shown to inhibit Ca^{2+} channels in other systems (13, 27, 28). The presence of 200 μM GTPγS in the patch electrode reduced the peak current amplitude in both UND and NGF-treated cells by 40–60%, compared with currents in cells intracellularly infused without GTPγS (data not shown). This reduction in current caused by infusion of GTPγS was much greater than the 10–15% reduction in current that occurred during normal rundown of Ca^{2+} current in both cell types. Results with GTPγS indicate that the activation of a G protein can modulate the Ca^{2+} currents in both cell types.

Because the inhibition of currents by intracellular infusion of GTPγS did not reach a steady state and continued to increase during the course of the experiments, similarly to

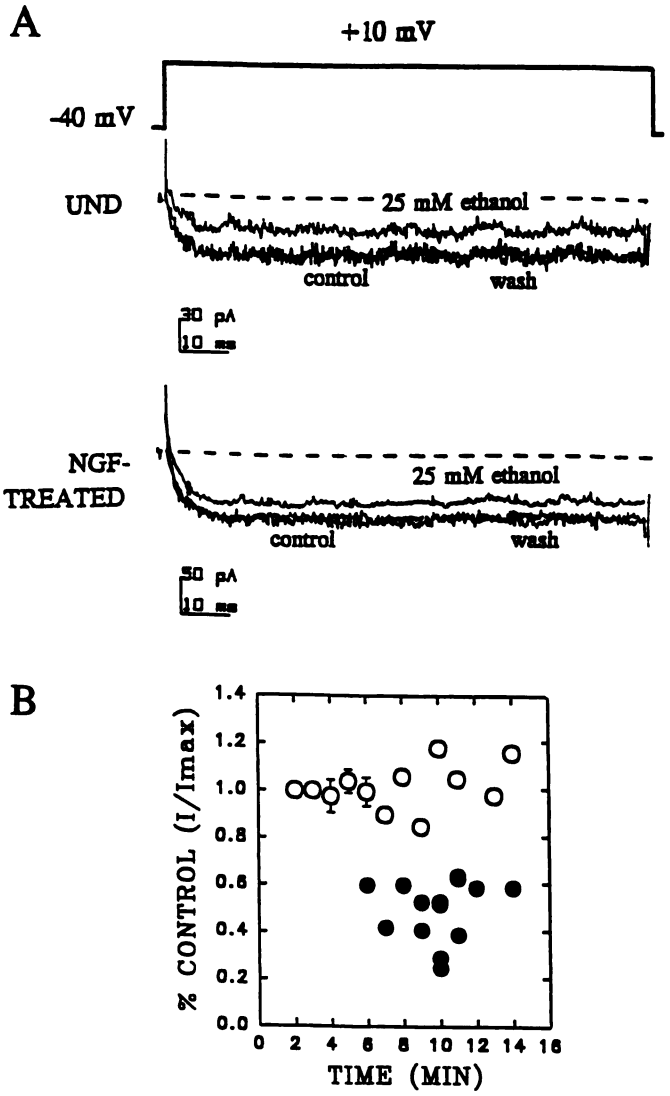

Fig. 1. A, Inhibition of Ca^{2+} currents by acute exposure to ethanol. Representative current traces in an UND cell (*top*) and in a NGF-treated cell (*bottom*) before (*control*), during, and after (*wash*) a 5-min superfusion with ethanol are shown. Note the different amplitudes of the scale bars. Peak current amplitude was determined by averaging all data points acquired between 20 and 80 msec of the 100-msec depolarization. B, Evidence that ethanol inhibition is not due to rundown of current. Normalized peak current in UND cells (I_{max} = 1) (mean ± standard error; individual points are noted without error bars, and for the 2- and 3-min time points in the absence of ethanol the error bars are smaller than the symbols) is plotted versus time after establishment of the whole-cell configuration, in the absence (○) and presence (●) of 25 mM ethanol. In the absence of ethanol, peak current was determined and all other current values at other time points were calculated as a fraction of peak current (percentage of control) (I/I_{max}). In the presence of ethanol, current values were calculated as a fraction of peak currrent in the absence of ethanol (percentage of control) (I/I_{max}).

results reported by Shapiro and Hille (13), we could not use this GTP analog to test for G protein involvement in ethanol inhibition. Instead, we used the nonhydrolyzable GDP analog GDPβS. This compound blocks activation of G proteins by competing for the GTP binding site. By stabilizing the G proteins in their inactive form, GDPβS has been shown to reduce the inhibitory effects of many neurotransmitters on Ca^{2+} channels (13, 29, 30). Shown in Fig. 2 are representative traces in which currents in a control UND cell (Fig. 2)

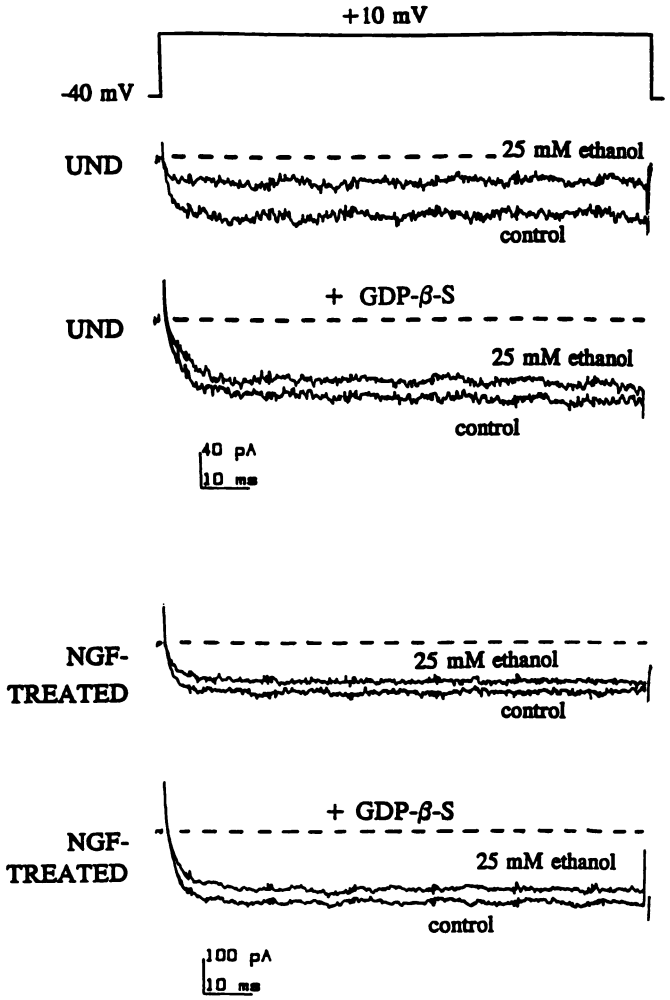

Fig. 2. GDPβS reduction of ethanol inhibition in UND cells but not in NGF-treated cells. Representative current traces obtained in the absence and presence of 25 mM ethanol in two UND and two NGF-treated cells, intracellularly infused in the absence and presence of 1 mM GDPβS, are shown.

and in an UND cell intracellularly infused with 1 mM GDPβS (Fig. 2) were decreased 55% and 18%, respectively, by 25 mM ethanol. The decrease in current produced by ethanol in control UND cells (average for group, 41.6 ± 6.6%; $n = 5$) was significantly ($p < 0.002$) greater than the decrease in current produced by ethanol during intracellular infusion with GDPβS (average for group, 10.7 ± 4.2%; $n = 6$). GDPβS had no effect on currents in the absence of ethanol. In contrast to the results in UND cells, GDPβS did not produce a decrease in ethanol inhibition in NGF-treated cells. After NGF treatment, ethanol inhibited current in a control cell by 23%, whereas current in a cell infused with 1 mM GDPβS was inhibited by 24% (Fig. 2). The decrease in current produced by ethanol in control NGF-treated cells (average for group, 27.7 ± 5.1%; $n = 7$) was not different from the reduction of current produced in NGF-treated cells intracellularly infused with GDPβS (average for group, 31.6 ± 7.8%; $n = 8$). Higher concentrations of GDPβS also had no effect on ethanol inhibition of current in these cells. Collectively, these results indicate that a G protein is involved in ethanol inhibition of Ca^{2+} currents in UND cells but does not appear to be involved in the inhibition in NGF-treated cells.

Identification of the G protein that modulates ethanol inhibition. Because many G proteins are present in PC-12 cells, we used PTX to help identify the family of G proteins involved in mediating ethanol inhibition. PTX is thought to prevent receptor activation of the G_i and G_o families of G proteins (31). PTX catalyzes the transfer of ADP-ribose from intracellular NAD^+ to the α subunits of the heterotrimeric complexes of G_i and G_o, thus altering the α subunits. Fig. 3 summarizes the effects of PTX pretreatment in both cell types. In UND cells treated with heat-inactivated PTX, ethanol inhibited currents by 34.3 ± 5.3% ($n = 6$). In UND cells pretreated with active PTX for 3 hr, ethanol inhibition was reduced only slightly, to 25.4 ± 8.7% ($n = 5$). Incubation of cells with PTX overnight produced a significant ($p < 0.02$) reduction in ethanol inhibition of current, to 11.1 ± 6.1% ($n = 7$). PTX pretreatment either for 5 hr or overnight did not reduce ethanol inhibition of currents in NGF-treated cells. These results demonstrate that the G protein involved in ethanol inhibition of Ca^{2+} currents in UND cells is sensitive to PTX.

The studies with PTX implicated the G_i/G_o class of G proteins as mediators of the effects of ethanol on L-type Ca^{2+} channels in PC-12 cells. This is a large class of G proteins with many members (31, 32). Several G_i/G_o-related G proteins that are PTX substrates have been specifically identified in PC-12 cells (16, 17, 19). These include G_{i2} and, less prominently, G_{i1}, as well as two isoforms of G_o referred to as

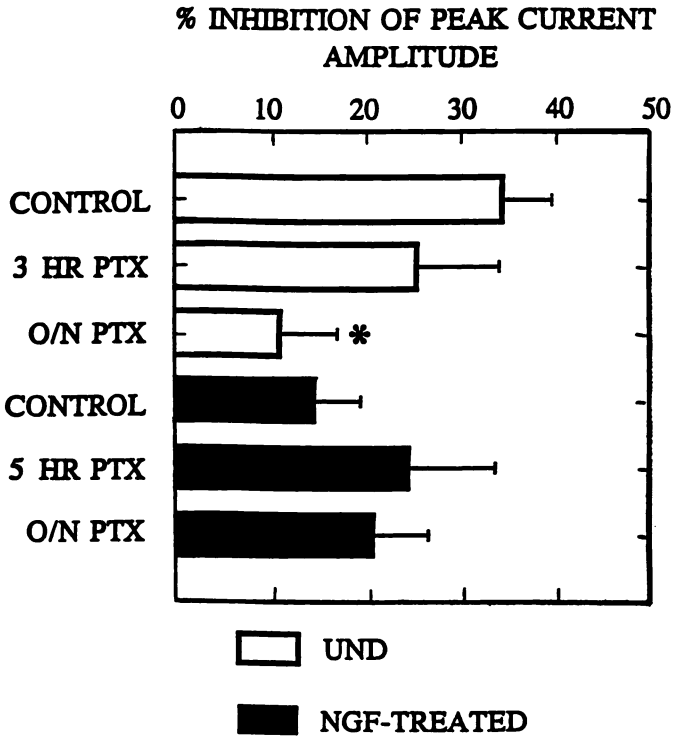

Fig. 3. Differential effects of PTX treatment on ethanol inhibition in UND and NGF-treated cells. Peak currents were evoked by a step to +10 mV from a holding potential of −40 mV, in the absence and presence of 25 mM ethanol, in UND and NGF-treated cells incubated with either 200 ng/ml heat-inactivated PTX (control) or 200 ng/ml active PTX for 3 hr, for 5 hr, or overnight (*O/N*) as noted. *, Ethanol inhibition was significantly ($p < 0.02$) reduced from control in UND cells treated with PTX overnight. A 5-hr or overnight treatment with PTX did not significantly alter ethanol inhibition in NGF-treated cells. From *top* to *bottom*, *n* values were 6, 5, 7, 6, 4, and 5.

G_{oA} and G_{oB}. To verify the presence of these G proteins and to determine the effect of treatment with NGF on the relative amounts of these proteins in our PC-12 cells, affinity-purified, site-specific antibodies were used on immunoblots of membranes prepared from both cell types (Fig. 4). These studies used antibodies specific for G_{i1} and G_{i2} (AI12) (which share an identical carboxyl terminus), G_{oA} (GOA), G_{oB} (GOB), and the β subunit (BC1) common to all G proteins. Because the α subunits, compared with the β subunits, may make only a small contribution to the total complement of G proteins present in cells, we used the antibody to the β subunit to assess the effect of NGF treatment on the complement of the major G proteins in the cells. The GOA and GOB antibodies recognized proteins of 39–40 kDa in both sets of membranes. Although GOA reacted strongly with bovine brain G proteins, GOB did not. This result is compatible with the previously reported specificity of the GOB antibody (17). Treatment of PC-12 cells with NGF resulted in a substantial increase in the amount of immunoreactive $G_{oA\alpha}$, as reported previously (17, 19). A similar but less pronounced increase in immunoreactive β subunit was seen with an antibody against the carboxyl terminus of G protein β subunits (BC1). Interestingly, NGF treatment resulted in opposite effects on the levels of immunoreactive $G_{oA\alpha}$ and $G_{oB\alpha}$, with the latter showing a definitive decrease in response to NGF treatment. These data are in contrast to those described by Asano *et al.* (17), who reported no change in the levels of $G_{oB\alpha}$. Different methods used or variations in the PC-12 cell clones used may underlie these dissimilar results.

The AI12 antibody recognized a single 40-kDa protein in membranes from both UND and NGF-treated cells. In contrast, the antibody recognized a doublet, corresponding to the α_{i1} and α_{i2} proteins, in the mixed preparation of G proteins purified from bovine brain (33). Others have reported a predominance of G_{i2} over the G_{i1} isoform in PC-12 cells (16, 18, 19). Our results are in agreement with this, but we cannot rule out the possibility that the cells may also contain low levels of α_{i1}. Qualitatively, there was a slight but noticeable increase in AI12 immunoreactivity after NGF treatment.

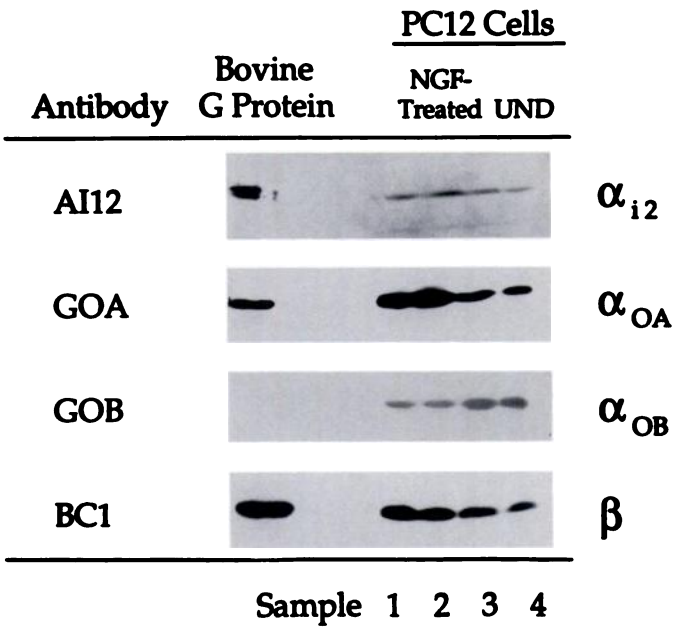

Fig. 4. Immunological analysis of NGF-induced changes in G protein subunit isoforms in PC-12 cells. Crude cell membranes were prepared from duplicate UND (samples 3 and 4) and NGF-treated (samples 1 and 2) PC-12 cell cultures. Fifty micrograms of membrane protein and 0.5 μg of purified mixed G proteins from bovine brain (as a positive control) were applied to an 11% sodium dodecyl sulfate-polyacrylamide gel. Proteins were identified with affinity-purified antibodies against G protein subunits; GOA recognizes α_{oA} (1/15,000 dilution), GOB recognizes α_{oB} (1/10,000 dilution), AI12 recognizes both α_{i1} and α_{i2} (1/700 dilution), and BC1 recognizes the β subunit of G proteins (1/10,000 dilution). Bound antibodies were visualized using enhanced chemiluminescence.

Use of antibodies to identify specific G proteins mediating the effect of ethanol on L-type Ca^{2+} channels. The availability of antibodies specific to the different PTX-sensitive G proteins in PC-12 cells suggested the possibility that they could be used to identify the specific G protein mediating the effects of ethanol. To do so, they would have to block the signaling pathway that occurs by way of these G proteins. To determine whether the AI12 antibody was capable of doing so, we monitored the effect of the antibody on the inhibition of currents produced by exposure to 10 μM clonidine. An antibody that recognizes the same sequence in the carboxyl termini of α_{i1} and α_{i2} as does the AI12 antibody has been used to show that G_{i2} mediates α_2-adrenergic receptor inhibition of adenylyl cyclase in platelet membranes (34). Clonidine is a selective agonist for the α_2-adrenergic receptor, which can couple to G_i or G_o, and this ligand has been shown to inhibit DHP-sensitive Ca^{2+} currents in UND PC-12 cells (16). Intracellular infusion of UND cells with the AI12 antibody reduced clonidine inhibition from 33.6 ± 10.9% (n = 3) to 10.7 ± 2.9% (n = 3). In the absence of clonidine, the AI12 antibody had no effect on currents. Similarly, we determined, by examining the inhibition of Ca^{2+} currents by DADLE, that the GOA and GOB antibodies blocked the function of the α_{oA} and α_{oB} proteins. The peptide DADLE is a selective agonist for the δ-opioid receptor, which can also couple to G_o or G_i, and has also been shown to reduce Ca^{2+} currents in UND PC-12 cells (16). Inhibition of currents by 1 μM DADLE was reduced from 28.1 ± 7.1% (n = 3) to 2.5% (0 and 5%; n = 2) by the GOB antibody. The GOA antibody did not affect inhibition of currents by DADLE. These results indicate that the AI12 and GOB antibodies can attenuate receptor-mediated inhibition of Ca^{2+} currents in UND PC-12 cells and can be used to determine which G protein is involved in mediating ethanol inhibition of Ca^{2+} currents. It is not clear whether the GOA antibody can block the α_{oA} protein in intact cells. Our finding that the antibody recognized the $G_{oA\alpha}$ protein in Western analysis suggests that the antibody is functional *in vitro*. Both GOA and GOB antibodies were raised against endogenous sequences in the respective G protein α subunits located in or near a region predicted to contain the effector binding site, based upon homology to the α subunit of transducin (35, 36), so we would expect them to block the function of the α_{oA} and α_{oB} proteins.

Using these same antibodies, we sought to identify which PTX-sensitive G protein mediated ethanol inhibition of current. In an UND cell infused with heat-inactivated AI12 antibody (Fig. 5, *top*), ethanol inhibited the current by 48% (average for group, 42.2 ± 2.5%; n = 11) (Table 1), which was not significantly different from the inhibition in the absence of inactivated antibody. In contrast, ethanol inhibition was significantly reduced ($p < 0.009$) to 17% (reduction seen in 11 of 12 cells; average for group, 16.4 ± 4.6%; n = 12) in an UND cell infused with active AI12 antibody (Fig. 5, *bottom*). In

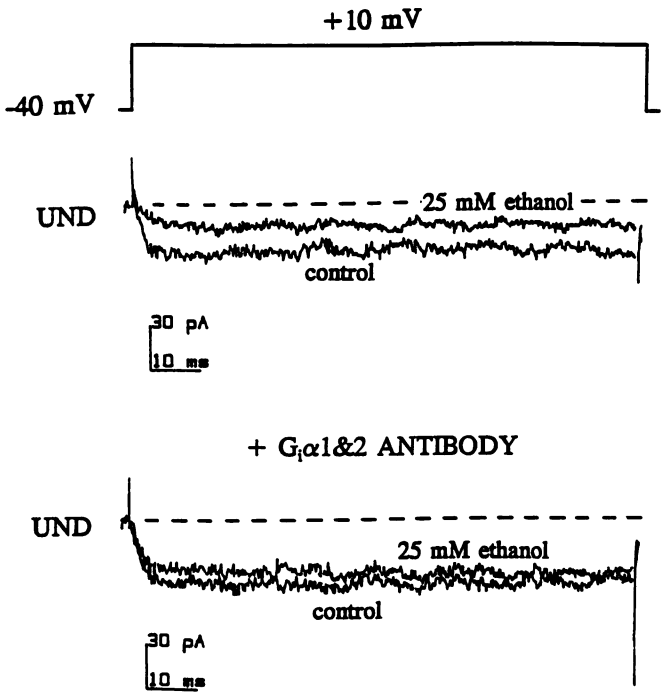

Fig. 5. AI12 antibody reduction of ethanol inhibition in UND cells. Representative current traces, in the absence and presence of 25 mM ethanol, in an UND cell intracellularly infused with heat-inactivated AI12 antibody (*top*) and in a different UND cell intracellularly infused with active AI12 antibody (*bottom*) are shown.

TABLE 1

Effects of AI12, GOA, and GOB antibodies on ethanol inhibition of peak current amplitude

Data are expressed as mean ± standard error (*n*, number of cells). The holding potential was −40 mV.

	Inhibition by 25 mM ethanol	
	Heat-inactivated	Active
	%	
UND cells		
AI12	42.2 ± 2.5 (*n* = 11)	16.4 ± 4.6 (*n* = 12)
GOA	46.5 (*n* = 2)	44.8 ± 16.0 (*n* = 5)
GOB	41.3 ± 12.5 (*n* = 3)	40.9 ± 8.7 (*n* = 4)
NGF-treated cells		
AI12	28.0 ± 5.9 (*n* = 3)	34.3 ± 4.5 (*n* = 3)
GOA	ND[a]	34.1 ± 8.8 (*n* = 3)
GOB	ND	59.2 ± 17.9 (*n* = 4)

[a] ND, not determined.

UND cells, neither heat-inactivated GOA or GOB nor active GOA or GOB antibodies had an effect on the currents or the response to ethanol, compared with control cells (Table 1). In NGF-treated cells, neither the AI12 antibody nor the GOA or GOB antibodies caused any decrease in ethanol inhibition (Table 1). In fact, the GOB antibody potentiated the ethanol inhibition of Ca^{2+} currents in these cells. The cause of this increase was not examined.

Evidence that G_i is not involved in the ethanol-induced shift in steady state inactivation of Ca^{2+} currents. Previously, we showed that acute exposure to ethanol caused a hyperpolarizing shift in the $V_{½}$ of L-type Ca^{2+} channels in both UND and NGF-treated PC-12 cells, contributing to the inhibition of L-type Ca^{2+} current (4). To determine whether the $G_{i1\alpha}$ or $G_{i2\alpha}$ proteins were involved in this action of ethanol, we intracellularly infused UND cells with the AI12 antibody and measured steady state inactivation of the current (Table 2). Using 10 mM Ca^{2+} as the charge carrier, currents were evoked from various holding potentials, before stepping to +10 mV, in the absence and presence of 25 mM ethanol. Data were normalized to the current obtained at a holding potential of −80 mV and the results were fitted using a curve-fitting routine, according to the Boltzmann equation. In the absence of ethanol, the $V_{½}$ was unaltered by intracellular infusion with either heat-inactivated or active antibody. Exposure to 25 mM ethanol caused an approximately 10-mV hyperpolarizing shift in the $V_{½}$ in the presence of either heat-inactivated AI12 antibody or active antibody. In addition, the slope values (k) were not different between control and ethanol or heat-inactivated antibody and active antibody treatments. The voltage dependence of the inactivation of the currents did not shift with time in the absence of any treatment ($V_{½}$ = −32.7 ± 1.3 mV at 6 min versus −33.8 ± 2.5 mV at 12 min, n = 3). These results with the AI12 antibody suggest that the ethanol-induced shift in steady state inactivation of Ca^{2+} channels, common to both UND and NGF-treated cells, is not mediated by the G_{i1} or G_{i2} protein.

Discussion

The results of this study indicate that a large part of the ethanol inhibition of DHP-sensitive, L-type Ca^{2+} current in UND PC-12 cells, but not in NGF-treated cells, is mediated by the PTX-sensitive G protein G_i. In addition, the PTX-sensitive G proteins G_{i1} and G_{i2} do not appear to be involved in the ethanol-induced shift in steady state inactivation of the channels in UND cells. These results suggest that ethanol can inhibit L-type Ca^{2+} channels by multiple mechanisms or pathways.

We found that intracellular infusion of GTPγS inhibited Ca^{2+} currents in both PC-12 cell types, similarly to the degree of inhibition shown in superior cervical ganglion neu-

TABLE 2

Evidence that the AI12 antibody does not alter the ethanol-induced shift in voltage dependence of steady state inactivation in UND cells

Currents were evoked, in the absence and presence of ethanol, from various holding potentials (−80, −70, −60, −50, −40, −30, and −20 mV), which were maintained for 30 sec before stepping to +10 mV for 50 msec. Data are presented as mean ± standard error (*n*, number of cells).

	Control		25 mM Ethanol	
	$V_{½}$	k	$V_{½}$	k
	mV	*mV*	*mV*	*mV*
Heat-inactivated antibody (*n* = 4)	−33.0 ± 0.9	6.3 ± 1.8	−43.1 ± 2.5	6.0 ± 0.8
Active antibody (*n* = 5)	−34.8 ± 1.8	9.0 ± 0.8	−43.6 ± 1.6	8.1 ± 1.0

rons (13, 37). These results indicate that activation of a G protein can result in inhibition of Ca^{2+} currents. In UND PC-12 cells, it appears that activation of G_i is required for ethanol inhibition of Ca^{2+} currents. Although infusion with GTPγS produced a decrease in current amplitude in NGF-treated cells, it appears that activation of a G protein is not involved in ethanol inhibition of current in these cells. In addition to its ability to activate the G protein involved in ethanol inhibition in UND cells, the ability of GTPγS to activate many G protein pools, which evoke a variety of effects on L-type channels (38), may explain why this analog caused a decrease in Ca^{2+} currents in both cell types. These effects, shown in cardiac ventricular myocytes, include GTPγS activation of Ca^{2+} channels by stimulation of G_s, interaction with a PTX-sensitive G protein (probably G_i), resulting in less agonist-induced activation of the channel, and enhancement of the response to cAMP-dependent phosphorylation, which could directly affect L-type channels.

In UND cells GDPβS reduced ethanol inhibition of Ca^{2+} current by ~75%, whereas in NGF-treated cells the analog caused no reduction in ethanol inhibition. To obtain conclusive evidence for the involvement of a G protein in UND cells, we used PTX, which is highly specific for the inhibitory G proteins G_i and G_o. This toxin reduced most of the ethanol inhibition in UND cells but did not diminish ethanol inhibition in NGF-treated cells, thus substantiating the results we obtained with GDPβS in both cell types. Our immunoblot results demonstrated the presence of PTX-sensitive G protein α subunits of the G_i and G_o families in both cell types and are in agreement with results shown by others (16, 17, 19). The fact that similar levels of G_i protein were found in both cell types suggests that the lack of G_i involvement in ethanol inhibition of current in NGF-treated cells cannot be accounted for by a decrease in levels of this protein. Our results, however, do not rule out the possibility that the G_i protein or the Ca^{2+} channel may have been altered by NGF treatment. If G_{oA} were involved in ethanol inhibition in NGF-treated cells, one would have expected to see a response to the GOA antibody, because the G_{oA} protein was greatly increased in these cells. The lack of an effect of the GOB antibody may have been due to the sizable decrease, compared with UND cells, in G_{oB} protein levels after NGF treatment. Because neither GDPβS nor PTX reduced ethanol inhibition in NGF-treated cells, it seems unlikely that a G protein is involved in ethanol inhibition of current in these cells. We are unaware of any known G proteins that do not respond to GDPβS. Of course, it is also possible that the Ca^{2+} channel is altered by NGF treatment such that it cannot participate in ethanol-modulated interactions with G_i.

The present study is the first direct demonstration of α_i regulation of DHP-sensitive Ca^{2+} channels. Our results are in agreement with those of Ewald *et al.* (11), who showed that G_i could partially reconstitute neuropeptide Y and bradykinin-induced inhibition of current; however, those investigators did not identify the Ca^{2+} channel type affected. In addition, receptor-mediated, hormonal inhibition of DHP-sensitive Ca^{2+} channels in UND PC-12 cells has been shown to be mediated by PTX-sensitive G proteins, but the identity of the G protein(s) involved was not determined (16). Our finding that the AI12 antibody attenuated clonidine inhibition of Ca^{2+} currents indicates that G_i is involved in α_2-adrenergic receptor-mediated as well as ethanol-mediated effects on L-type Ca^{2+} channels. These results with clonidine are in agreement with those of Simonds *et al.* (34), who used an antibody that recognizes the same sequence in the carboxyl terminus of α_{i1} and α_{i2} as does our AI12 antibody and showed that their antibody blocked α_2-adrenergic receptor-mediated inhibition of adenylyl cyclase in crude platelet membranes. Other investigators found that infusion with the purified α subunit of G_o restored DADLE inhibition of Ca^{2+} currents in differentiated NG108–15 cells (39). Our results with the GOB antibody indicate that the G_{oB} protein functionally couples the δ-opioid receptor to Ca^{2+} channels in UND PC-12 cells.

It is likely that under certain conditions other G proteins may mediate ethanol inhibition of Ca^{2+} currents in UND cells. Indeed, it has been shown that, for bradykinin, combinations of purified G proteins are required to restore peptide-induced inhibition of currents in PTX-treated dorsal root ganglion neurons (11). In two UND cells, the GOB antibody was able to reduce ethanol inhibition of current almost as well as the AI12 antibody (data not shown). It is possible that the G_{oB} α subunit can sometimes substitute for the G_i α subunit and thus mediate ethanol inhibition of Ca^{2+} currents. Interestingly, in NGF-treated cells, infusion with either the GOA or AI12 antibody caused a slight potentiation of ethanol inhibition, whereas the GOB antibody caused a much larger potentiation. Because neither the antibodies nor GDPβS caused an increase in currents in the absence of ethanol, it is unlikely that the channels in either cell type are tonically inhibited by the G proteins. It is unclear what might underlie the potentiation of ethanol inhibition in NGF-treated cells.

Because neither GDPβS nor PTX reduced ethanol inhibition in NGF-treated cells, it is unlikely that G proteins are involved in ethanol inhibition of currents in these cells. However, our results with GTPγS suggest that inhibition of channel activity by G proteins can occur in these cells, and our immunoblot studies indicate that G_i is, indeed, present in the NGF-treated cells. Because G_i may be the only G protein capable of ethanol-mediated G protein interaction with L-type channels in UND PC-12 cells, it is possible that after NGF treatment G_i is prevented from interacting with L-type channels in these cells. Recently, NGF treatment of PC-12 cells has been shown to induce changes in the localization of G protein subunits, in particular $G_{i\alpha}$ and $G_{o\alpha}$, within the cells (19). A redistribution of $G_{i\alpha}$ after NGF treatment, essentially making $G_{i\alpha}$ unavailable, may be the most likely explanation for the lack of G protein involvement in ethanol inhibition of current in these cells. Alternatively, the channel may be modified in a manner that precludes ethanol-mediated alterations in its interactions with G_i.

In most UND cells, block of ethanol inhibition by the AI12 antibody, GDPβS, or pretreatment with PTX was not complete. The contribution of the ethanol-induced shift in steady state inactivation could account for this incomplete effect. The finding that the AI12 antibody did not affect the ethanol-induced shift in steady state inactivation suggests that G proteins may not be involved in the voltage dependence of inactivation of Ca^{2+} channels in PC-12 cells. Our previous findings (4) indicated that ethanol had a greater effect on the inactivated state than on the resting or activated state of Ca^{2+} channels and that a portion of ethanol inhibition could be ascribed to a hyperpolarizing shift in steady state inacti-

vation. A recent hypothesis (40) suggests that activated G proteins preferentially interact with Ca^{2+} channels in the resting state, resulting in a modified state that may be unavailable for opening upon depolarization. Further analysis at the single-channel level will be required to determine how ethanol interacts with the inactivated state of the channel and whether a G protein is involved in this interaction.

Thus far, the mechanisms, which may be direct or indirect, underlying neurotransmitter-induced decreases in voltage-dependent Ca^{2+} currents have been shown to be varied; some involve protein phosphorylation by protein kinase C (41, 42) or cAMP-dependent protein kinase (43), whereas others involve the well characterized signal transduction pathway belonging to G proteins (10–13, 16, 27–29, 44–46). A recent study suggests that, in murine sensory neurons, inhibition of Ca^{2+} channels by acute exposure to ethanol requires activation of G_o (47). Determination of whether Ca^{2+} channels and the effects of acute exposure to ethanol are modulated directly or indirectly by G proteins must await examination using either purified or cloned channels and G proteins reconstituted into defined lipid bilayers.

Collectively, our results indicate that the PTX-sensitive G protein G_i is involved in ethanol inhibition of L-type Ca^{2+} channels in UND cells but not in NGF-treated cells. We propose that the difference in ethanol sensitivity of the Ca^{2+} channels in the two cell types is due to the involvement of G_i in UND cells and not in NGF-treated cells. To the best of our knowledge, this is the first time that G_i has been shown to be involved in DHP-sensitive, L-type Ca^{2+} channel inhibition.

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