

# Protein Kinase C $\epsilon$ Mediates Up-Regulation of N-Type Calcium Channels by Ethanol

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## ABSTRACT

Brief exposure to ethanol inhibits L-type and N-type voltage-gated calcium channels in neural cells. Although chronic ethanol exposure up-regulates the density and function of L-type channels via a protein kinase C (PKC)  $\delta$ -dependent mechanism, the effect of prolonged ethanol exposure on N-type channels is not known. Using PC12 cells, we found that exposure to 25 to 150 mM ethanol for 0 to 8 days produced a time- and concentration-dependent increase in the density of binding sites for the N-type channel antagonist  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA. This was associated with an increase in  $\omega$ -conotoxin GVIA-sensitive, depolarization-evoked rises in  $[\text{Ca}^{2+}]_i$ . Increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding also were observed in the frontal cortex

and the hippocampus, but not in the thalamus of mice exposed to ethanol vapor for 3 days. In PC12 cells, increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding were blocked by the PKC inhibitor bisindolylmaleimide I and by expression of a selective peptide inhibitor of PKC $\epsilon$ . Expression of a selective inhibitor of PKC $\delta$  did not alter ethanol-induced increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding. These findings indicate that PKC $\epsilon$  mediates up-regulation of N-type channels by ethanol. Because N-type channels modulate calcium-dependent neurotransmitter release, these findings suggest a mechanism that may contribute to neuronal hyperexcitability observed during alcohol withdrawal.

Voltage-gated  $\text{Ca}^{2+}$  channels mediate  $\text{Ca}^{2+}$  entry into neurons and regulate firing patterns, neurotransmitter release, gene expression, and differentiation (Dunlap et al., 1995; Ghosh and Greengard, 1995). Several manifestations of ethanol intoxication and dependence may be due to modulation of  $\text{Ca}^{2+}$  channel function. In nerve terminals from rat neurohypophysis and in PC12 cells, brief exposure to intoxicating concentrations (10–50 mM) of ethanol inhibits L-type channels by decreasing open channel probability (Wang et al., 1994), promoting channel inactivation (Mullikin-Kilpatrick and Treistman, 1995), and interacting with  $\text{G}_i$  (Mullikin-Kilpatrick et al., 1995). In addition to inhibiting L-type channels, recent evidence indicates that ethanol also inhibits N-type channels. In rat neurohypophysis, ethanol (50–100 mM) reduces N-type currents by 30 to 40% (Wang et al., 1991). Using PC12 cells, we recently found that 10 to 50 mM ethanol inhibits both N-type and Q-type channels by a mechanism that is antagonized by activating protein kinase A (Solem et al., 1997). In rat striatal synaptosomes, 200 mM ethanol inhibits ~65% of depolarization-evoked,  $\omega$ -conotoxin GVIA-sensitive dopamine release, suggesting that ethanol

also inhibits N-type channels in this preparation (Woodward et al., 1990). Inhibition of N channels may contribute to sedative effects of ethanol because intraventricular administration of  $\omega$ -conotoxin GVIA prolongs the duration of the loss of righting reflex induced by ethanol (Brown et al., 1993).

Much evidence indicates that chronic ethanol exposure increases the density and function of neuronal L-type channels. Prolonged exposure of PC12 cells to ethanol produces a reversible concentration- and time-dependent increase in L-type  $\text{Ca}^{2+}$  currents and in  $\text{K}^+$ -evoked  $^{45}\text{Ca}^{2+}$  uptake through L-type channels measured in the absence of ethanol (Messing et al., 1986; Grant et al., 1993). This is associated with a corresponding increase in the number of binding sites for dihydropyridine (DHP)  $\text{Ca}^{2+}$  channel antagonists. Similar increases in DHP binding have been detected in brain membranes from ethanol-dependent rodents (Brennan et al., 1990; Little, 1991). Up-regulation of L-type  $\text{Ca}^{2+}$  channels contributes to neuronal hyperexcitability observed during alcohol withdrawal because L channel antagonists reduce tremors, seizures, and mortality in alcohol-dependent rodents deprived of ethanol (Little et al., 1986; Bone et al., 1989). Increases in L-type channels also may promote alcohol consumption because L-type channel antagonists reduce ethanol self-administration in animals (Rezvani et al., 1991; Fadda et al., 1992).

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Protein kinase C (PKC) is a multigene family of 10 phospholipid-dependent, serine-threonine kinases central to many signal transduction pathways (Nishizuka, 1992). In chick skeletal myocytes (Navarro, 1987) and *Aplysia* bag cell neurons (Strong et al., 1987), phorbol esters that activate most PKC isozymes increase the number of functional  $\text{Ca}^{2+}$  channels. We found in PC12 cells that up-regulation of L-type channels by ethanol also is mediated by PKC because it is prevented by culturing cells with PKC inhibitors (Messing et al., 1990; Gerstin et al., 1998). Because ethanol increases L-type channels by a PKC-dependent mechanism, we investigated whether ethanol activates PKC. We found that chronic exposure to ethanol increases total PKC activity, high-affinity phorbol ester binding and PKC-mediated phosphorylation (Messing et al., 1991). This is associated with a selective increase in immunoreactivity (Messing et al., 1991) and mRNA (Roivainen et al., 1994) for two PKC isozymes, PKC $\delta$  and PKC $\epsilon$ . Expression of a fragment of PKC $\delta$  that antagonizes phorbol ester-induced translocation of this isozyme inhibits ethanol-induced increases in L channel density and function in PC12 cells (Gerstin et al., 1998). These findings demonstrate that PKC $\delta$  is specifically required for up-regulation of L-type channels by chronic exposure to ethanol.

Despite this wealth of data on L-type channels and ethanol, very little is known about N-type channels following chronic ethanol exposure. In this study, we examined the density and function of N-type calcium channels following chronic exposure to ethanol. Channel density was studied by measuring binding of the selective N-type channel antagonist  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA. The function of N-type channels was assayed by measuring depolarization-evoked rises in  $[\text{Ca}^{2+}]_i$  in the presence and absence of  $\omega$ -conotoxin GVIA. Finally, the dependence of these changes on PKC was examined by using cell lines that express PKC isozyme-selective inhibitor peptides. Our results provide evidence that chronic exposure to ethanol increases the density of N-type channels via a PKC $\epsilon$ -dependent mechanism.

## Experimental Procedures

**Materials.**  $^{125}\text{I}$ - $\omega$ -Conotoxin GVIA (2200 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). Fura-2 AM and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Bisindolylmaleimide I was purchased from Calbiochem Corp. (La Jolla, CA).

**Cell Culture.** PC12 cell lines were cultured in ethanol as described previously (Hundle et al., 1997; Gerstin et al., 1998). The generation and characterization of cell lines stably expressing  $\delta\text{V1}$  and  $\epsilon\text{V1}$  peptide fragments are described elsewhere (Hundle et al., 1997; Gerstin et al., 1998).

**Exposure of Mice to Ethanol Vapor.** Male mice were rendered physically dependent on ethanol in inhalation chambers as described previously (Terdal and Crabbe, 1994). Animal care and handling procedures were in accordance with institutional and National Institutes of Health guidelines. Mice were housed in cages suspended in Plexiglas chambers and ethanol was introduced into the chambers from a reservoir by a continuously operating peristaltic pump. Ethanol concentrations were adjusted by modulating the flow rate and the air/ethanol vapor was replaced approximately every 10 min. Ethanol concentrations were 7 to 10 mg ethanol/l air, adjusted daily to maintain blood ethanol concentrations at ~1.5 to 2.0 mg/ml. Mice were injected i.p. with a priming dose of ethanol (1.5 g/kg; 20% v/v in 0.9% saline) and pyrazole hydrochloride (1.0 mmol/kg) before being

placed in the inhalation chambers on day 1. At 24 and 48 h, the mice were briefly removed from the chambers, weighed, and given injections of pyrazole. Some mice had 20- $\mu\text{l}$  blood samples taken from the tail to monitor blood ethanol concentrations by gas chromatography. All mice were then replaced in the chambers. On withdrawal from the chambers (at 72 h), a tail blood sample was taken from each mouse and analyzed. Some control animals were injected with pyrazole, but received an i.p. saline injection instead of an ethanol loading dose, and were placed in identical chambers where they were exposed only to air. Additional control mice were given only saline injections and exposed to air.

**$^{125}\text{I}$ - $\omega$ -Conotoxin GVIA Binding.** Binding of  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA (80 pM) to intact PC12 cells was performed as described (Solem et al., 1997). Binding to brain membranes (0.5–1.5  $\mu\text{g}$ ) was measured as described by Wagner et al. (1988), except that all buffers contained the protease inhibitors phenylmethylsulfonyl fluoride (0.2 mM), benzamidine (0.1 mg/ml), pepstatin A (1  $\mu\text{g}/\text{ml}$ ), aprotinin (1.0  $\mu\text{g}/\text{ml}$ ), and leupeptin (1  $\mu\text{g}/\text{ml}$ ), and tissue was incubated with ligand in the presence of 0.1% BSA. Specific binding was calculated as total binding minus binding measured in the presence of 500 nM  $\omega$ -conotoxin GVIA and increased linearly with 0.5 to 2  $\mu\text{g}$  of brain tissue.

**Measurement of  $[\text{Ca}^{2+}]_i$ .** PC12 cells ( $5 \times 10^5$ ) were plated on 15-mm-diameter glass coverslips (Warner Instruments, Hamden, CT) pretreated for 30 min with 10% HCl in ethanol, washed in PBS, incubated with 0.1 mg/ml of poly-L-ornithine for 30 min, and coated with laminin (30  $\mu\text{g}/\text{ml}$ ) overnight at 37°C. The cells were grown for 6 days in culture medium (Dulbecco's modified Eagle's medium, 10% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin) in the presence or absence of 120 mM ethanol. Cells were rinsed twice in 5 mM KCl buffer (85 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 45 mM choline chloride, 5 mM glucose, 25 mM HEPES, pH 7.4) and incubated in 5 mM KCl buffer containing 10  $\mu\text{M}$  fura-2 AM and 0.02% Pluronic F-127 for 30 min on ice. The buffer was then removed and cells were incubated in 5 mM KCl buffer at 37°C for 15 min. The coverslip was mounted on a perfusion chamber (model RC-20; Warner Instruments) and perfused with 5 mM KCl buffer at 27°C. All subsequent procedures were performed at 27°C. Because millimolar concentrations of  $\text{Ca}^{2+}$  inhibit binding of  $\omega$ -conotoxin GVIA to N-type channels, cells were preincubated for 5 min in the presence or absence of 1  $\mu\text{M}$   $\omega$ -conotoxin GVIA in buffer containing 140 mM NaCl, 5 mM KCl, 12 mM glucose, 1  $\mu\text{M}$   $\text{CaCl}_2$ , 10 mM HEPES, pH 7.4, and 1 mg/ml BSA. Cells were subsequently incubated for 5 min in 5 mM KCl buffer and depolarized by incubation in 50 mM KCl buffer in the continued presence or absence of  $\omega$ -conotoxin GVIA. The 50 mM KCl buffer was similar in composition to 5 mM KCl buffer except that KCl was substituted for choline chloride. Images were captured and  $[\text{Ca}^{2+}]_i$  was calculated as described previously (Solem et al., 1997).  $R_{\text{max}}$  values were obtained by incubating cells for 10 min in 10 mM NaCl, 110 mM KCl, 10 mM EGTA, 10 mM  $\text{MgCl}_2$  and 5  $\mu\text{M}$  ionomycin.  $R_{\text{max}}$  was subsequently measured by incubating cells for 5 min in 10 mM NaCl, 110 mM KCl, 20 mM  $\text{CaCl}_2$ , and 5  $\mu\text{M}$  ionomycin. All buffers were added by gravity perfusion (Solem et al., 1997).

## Results

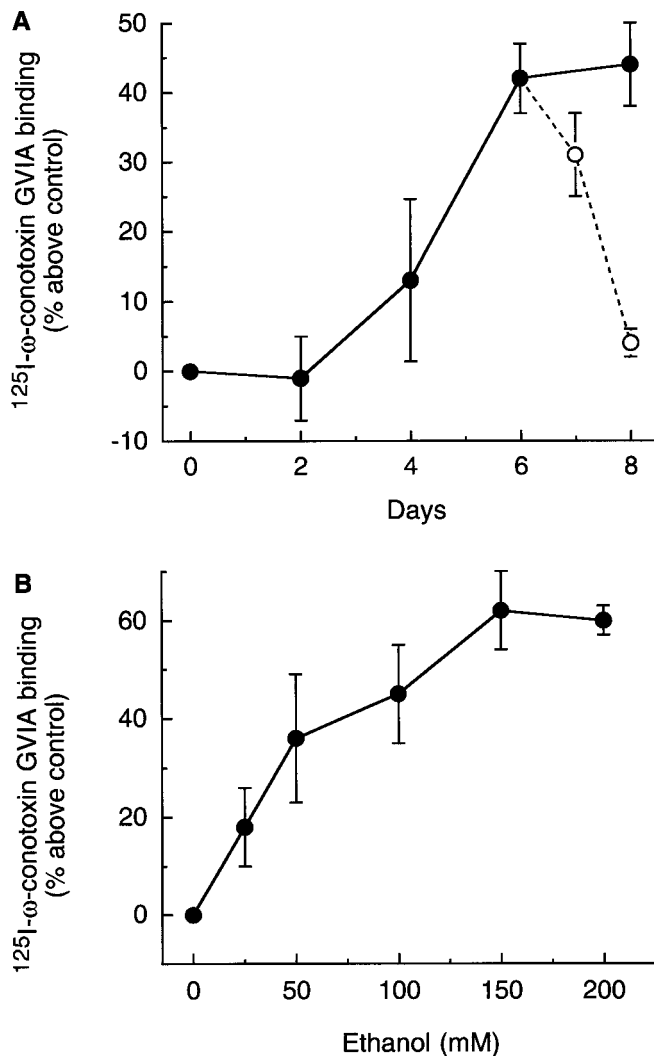
**Chronic Ethanol Exposure Increases  $^{125}\text{I}$ - $\omega$ -Conotoxin GVIA Binding in PC12 Cells.** To examine whether chronic ethanol exposure alters N channel density, we measured binding of  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA to intact PC12 cells after exposure to 25 to 150 mM ethanol for 1 to 8 days. Exposure to 100 mM ethanol evoked a time-dependent increase in binding that was maximal after 6 days of exposure (Fig. 1A). In cells treated with ethanol for 6 days, there was a concentration-dependent increase in binding that was maximal at  $60 \pm 17\%$  with an ethanol concentration of 150 mM

(Fig. 1B). Scatchard analysis of equilibrium saturation binding with 5 to 200 pM radioligand (Fig. 2A) revealed that exposure to 120 mM ethanol for 6 days increased the maximal number of binding sites for  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA from  $9.2 \pm 1.2$  fmol/mg in control cells to  $14.2 \pm 1.5$  fmol/mg in ethanol-treated cells ( $P < .028$ ;  $n = 5$ ), without altering binding affinity ( $K_D = 75.4 \pm 9.6$  pM in control and  $78.0 \pm 12$  pM in ethanol-treated cells;  $P = .87$ ;  $n = 5$ ). Ethanol-induced increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding were reversible (Fig. 1A); after 6 days of exposure to 120 mM ethanol, binding returned to baseline values 48 h after removal of ethanol.

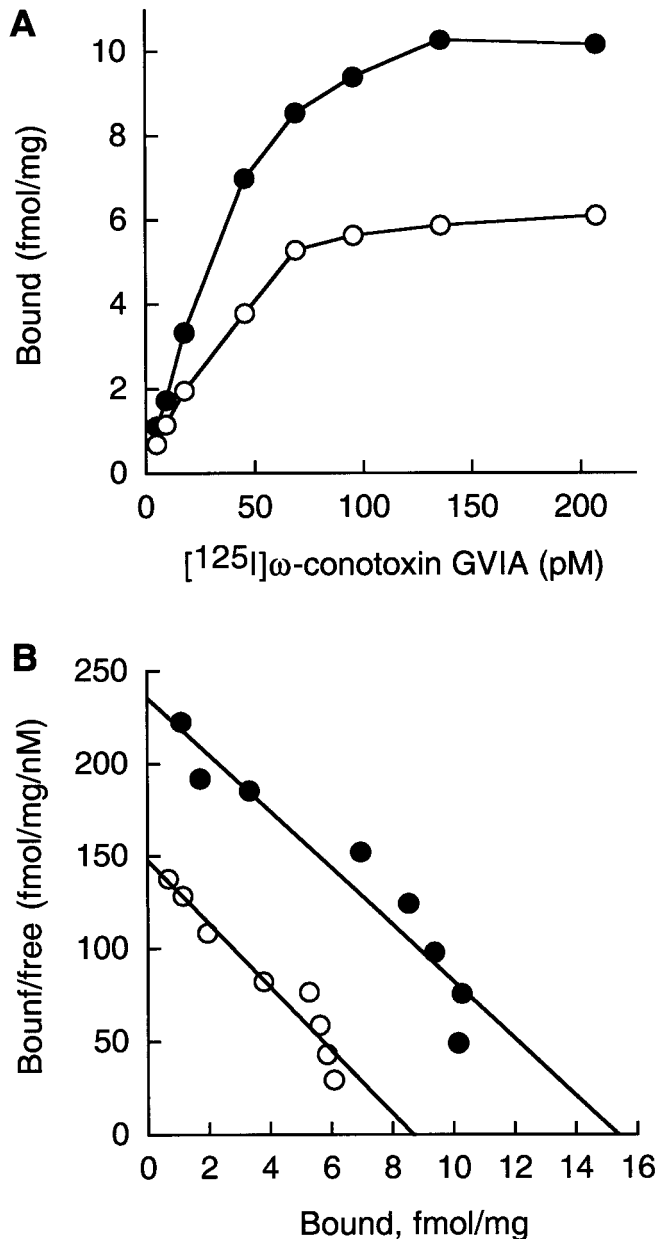
**Ethanol Increases  $\omega$ -Conotoxin GVIA-Sensitive Rises in  $[\text{Ca}^{2+}]_i$ .** To examine whether increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding reflect increases in functional N-type channels, we examined depolarization-induced rises in  $[\text{Ca}^{2+}]_i$  in cells loaded with fura-2. In control cells, depolarization with 50 mM KCl stimulated a rise in  $[\text{Ca}^{2+}]_i$  that was maximal 12 s after initiation of the infusion (Fig. 3A). Treat-

ment with  $\omega$ -conotoxin GVIA reduced the peak rise in  $[\text{Ca}^{2+}]_i$  by approximately one-third (Fig. 3B). In cells cultured with 120 mM ethanol for 6 days, depolarization evoked a similar peak rise in  $[\text{Ca}^{2+}]_i$  but this response was more sensitive to  $\omega$ -conotoxin GVIA, which inhibited the rise by  $\sim 68\%$  in ethanol-treated cells (Fig. 3, A and B). This indicates that depolarization-evoked peak rises in  $[\text{Ca}^{2+}]_i$  in PC12 cells are more dependent on  $\text{Ca}^{2+}$  influx through N-type channels following chronic ethanol exposure.

**$^{125}\text{I}$ - $\omega$ -Conotoxin GVIA Binding in Brains of Mice Exposed to Ethanol Vapor.** When mice are exposed to ethanol vapor for 3 days, the density of binding sites for L-type calcium channel antagonists is increased in brain tissue



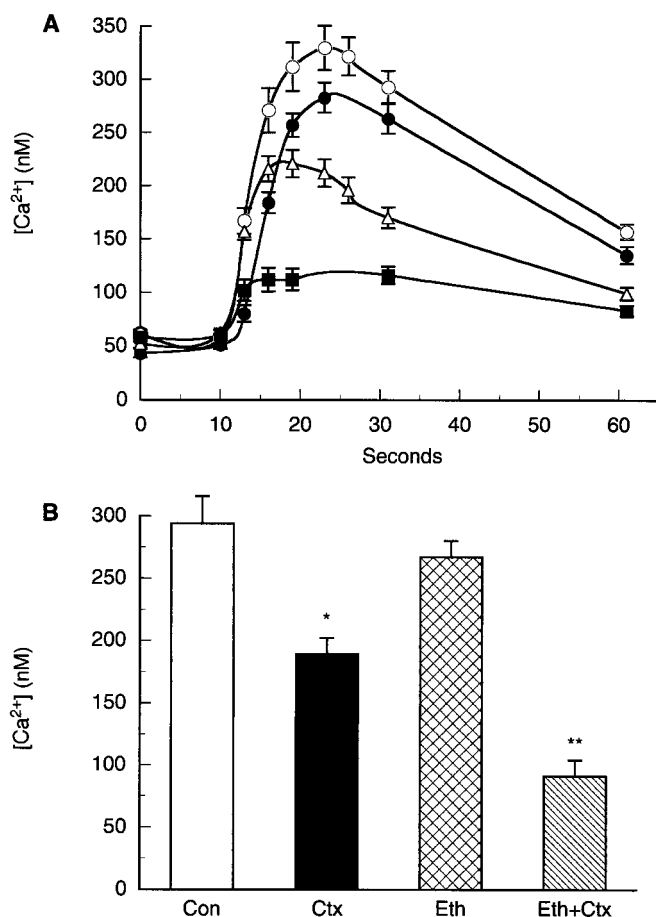
**Fig. 1.** Time course and concentration-dependence of increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding following exposure to ethanol. A, binding was measured in PC12 cells cultured with 120 mM ethanol ( $\bullet$ ). Some cultures were treated with ethanol for 6 days and then cultured for 1 to 2 days longer without ethanol ( $\circ$ ). B, binding was measured in PC12 cells cultured for 6 days in the indicated concentrations of ethanol. Data are means  $\pm$  S.E. ( $n = 8-16$ ) and are expressed as the percentage above binding measured in parallel control cells cultured without ethanol.



**Fig. 2.** Saturation analysis of specific  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding in PC12 cells. Data from saturation isotherms (A) were converted to Scatchard plots (B) and values for  $K_D$  ( $-1/\text{slope}$ ) and  $B_{max}$  ( $x$ -intercept) were determined by linear regression analysis. Data shown are from a representative experiment performed in triplicate. Mean  $K_D$  and  $B_{max}$  values  $\pm$  S.E. from five experiments are given in the text.

(Brennan et al., 1990). To examine if binding sites for N-type channels also are increased, we examined  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding in brains of mice exposed to ethanol by inhalation. For these studies, we used Withdrawal Seizure-Prone mice, which readily develop handling-induced convulsions and increases in L-type channels following chronic exposure to ethanol (Brennan et al., 1990). Binding was determined in samples from hippocampus, frontal cortex, and thalamus. To obtain stable concentrations of ethanol in the blood, control and ethanol-exposed mice were treated with daily injections of pyrazole. Initial studies revealed that pyrazole injection alone had no effect on  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding in all three brain regions ( $P > .17$ ). Therefore, we pooled data for control animals treated with and without pyrazole. In ethanol-treated mice, the mean blood ethanol level was  $1.24 \pm 0.20$  mg/ml ( $28 \pm 5$  mM), which is a concentration associated with moderate intoxication in humans. Ethanol treatment increased  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding in the hippocampus and the frontal cortex, but not in the thalamus (Fig. 4). These results suggest that ethanol increases  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding in specific brain regions.

**PKC Regulation of N-Type Channels by Chronic Ethanol Exposure.** Because ethanol increases the density of



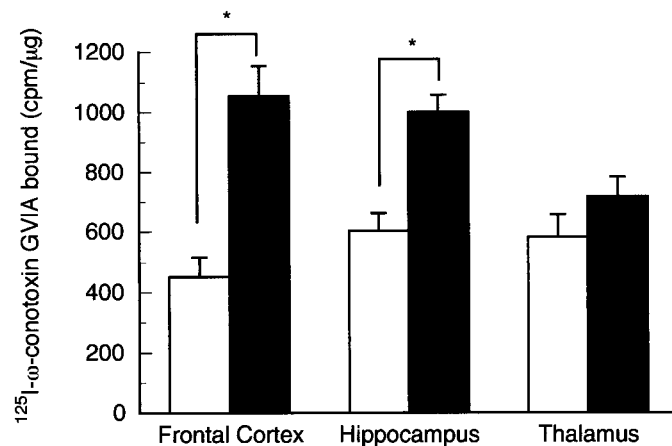
**Fig. 3.** Inhibition of depolarization-evoked rises in  $[\text{Ca}^{2+}]_i$  by  $\omega$ -conotoxin GVIA. PC12 cells were treated with (●, ■) or without (○, △) 120 mM ethanol for 6 days and then incubated in the presence (△, ■) or absence (○, ●) of 1  $\mu\text{M}$   $\omega$ -conotoxin GVIA before depolarization. A, time course of rises in  $[\text{Ca}^{2+}]_i$ . Perfusion with 50 mM KCl buffer was begun at the 10-s time point. B, maximal increases in  $[\text{Ca}^{2+}]_i$  for each condition. Data are means  $\pm$  S.E. from 86 (Con, ○), 166 (Ctx, △), 77 (Eth, ●), and 90 (Eth + Ctx, ■) cells in three experiments. \* $P < .0001$  compared with Con and \*\* $P < .0001$  compared with Con, Ctx, or Eth (ANOVA and Scheffe  $F$  test).

L-type channels by a PKC-dependent mechanism (Gerstin et al., 1998), we examined whether up-regulation of N-type channels by ethanol is also PKC-dependent. Bisindolylmaleimide I, which inhibits most PKC isozymes (Toullec et al., 1991), inhibited ethanol-induced increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding (Fig. 5A). Because ethanol selectively up-regulates PKC $\delta$  and PKC $\epsilon$  in PC12 cells (Messing et al., 1991), we investigated whether increases in  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding require PKC $\delta$  or PKC $\epsilon$ . We used PC12 cell lines that express the peptide fragments  $\delta\text{V1}$  or  $\epsilon\text{V1}$ , which selectively inhibit phorbol ester-stimulated translocation of PKC $\delta$  or PKC $\epsilon$ , respectively (Johnson et al., 1996; Hundle et al., 1997). Ethanol increased  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding to a similar extent in the parent cell line and in cells expressing the empty vector or  $\delta\text{V1}$  (Fig. 5B). However, in cells expressing  $\epsilon\text{V1}$ , ethanol did not increase  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding. These findings suggest that ethanol up-regulates N-type channels in PC12 cells by a PKC $\epsilon$ -dependent mechanism.

## Discussion

Our results are the first to indicate that chronic ethanol exposure increases N-type  $\text{Ca}^{2+}$  channels in neural tissues. Two previous studies suggested that chronic ethanol exposure does not increase N-type channel density or function. Woodward et al. (1990) examined depolarization-induced dopamine release from striatal synaptosomes and found that 500 nM  $\omega$ -conotoxin GVIA reduced release to a similar extent (36–44%) in synaptosomes from control rats and rats fed ethanol by liquid diet for 6–8 weeks. Bergamaschi et al. (1995) examined ethanol-treated NG108–15 cells and found that exposure to 200 mM ethanol for 72 h did not alter  $^{125}\text{I}$ - $\omega$ -conotoxin GVIA binding. The discrepancy between these results and ours may reflect differential sensitivity of N-type channels to ethanol in various brain regions and cell lines.

Our findings also indicate that in PC12 cells, ethanol increases N-type channels by a PKC $\epsilon$ -dependent mechanism. This is very different from ethanol-induced increases in L-type channels, which require PKC $\delta$ , not PKC $\epsilon$  (Gerstin et al., 1998). Both L-type and N-type channels are multimeric com-



**Fig. 4.**  $^{125}\text{I}$ - $\omega$ -Conotoxin GVIA binding in brain tissue from ethanol vapor-treated mice. Mice were exposed to air (□) or ethanol vapor (■) for 3 days and then brains were taken to measure binding in frontal cortex, hippocampus, and thalamus. Data are means  $\pm$  S.E. from 9 control and 15 ethanol-treated mice. \* $P = .002$  by two-tailed Student's  $t$  test.



plexes containing at least three types of subunits, and two of these,  $\beta$  and  $\alpha_2\delta$ , contribute to both classes of channels (Dunlap et al., 1995). In contrast, the type of  $\alpha_1$ -subunit is unique for each class. Thus, it is likely that ethanol-induced increases in N-type and L-type channels involve distinct mechanisms, one requiring PKC $\epsilon$ -mediated increases in N-type  $\alpha_{1B}$ -subunits, and another requiring PKC $\delta$ -mediated increases in L-type  $\alpha_{1C}$ - or  $\alpha_{1D}$ -subunits. This could involve PKC isozyme-mediated changes in subunit gene expression, mRNA stability, protein turnover, or protein trafficking. Recruitment of functional N-type channel complexes has recently been observed in cultured neural cells exposed to  $\omega$ -conotoxin GVIA for several hours (Passafaro et al., 1994) or to a variety of secretagogues, including KCl, ionomycin, and phorbol ester (Passafaro et al., 1996). Therefore, it is possible that ethanol-induced changes in protein trafficking could increase the density of functional N-type channels. This could specifically involve PKC $\epsilon$  because, upon activation, this PKC isozyme binds  $\beta'$ COP, a cotamer protein involved in vesicular trafficking (Csukai et al., 1997).

Brief exposure to alcohol (Solem et al., 1997), opiates (Soldo and Moises, 1997), or cannabinoids (Mackie and Hille, 1992) inhibits N-type channels, suggesting that N-type channels may be a common target for several drugs of abuse.

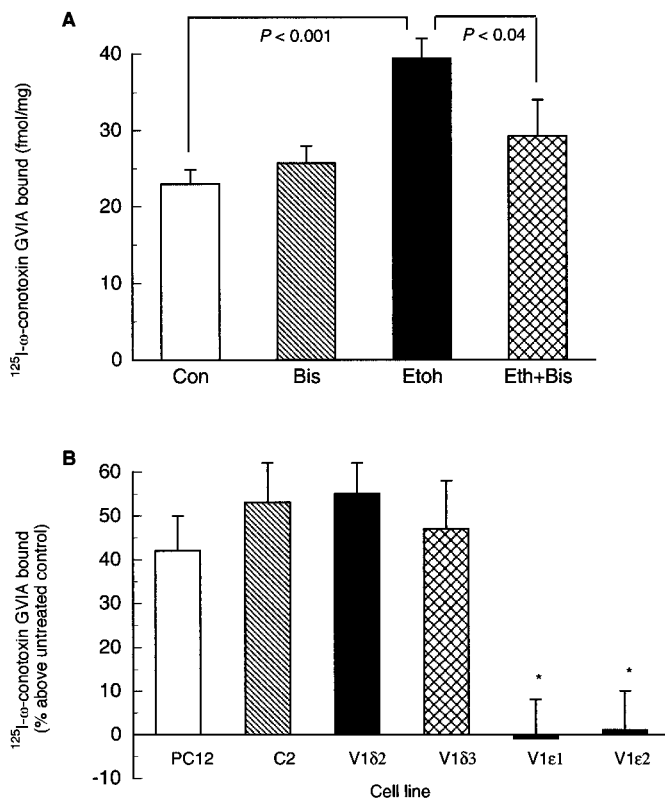
N-Type channels interact directly with the presynaptic protein synaptotagmin (Sheng et al., 1997) and with the synaptic core complex of proteins that regulate vesicle docking and membrane fusion during neurotransmitter release (Sheng et al., 1996). Because N-type channels regulate neurotransmitter release at several synapses (Dunlap et al., 1995), ethanol-induced increases in these channels may account for increases in transmitter release observed following chronic ethanol exposure (Nestby et al., 1997; Imperato et al., 1998). In addition, increases in N-type channels could promote neuronal hyperexcitability and contribute to manifestations of alcohol dependence and withdrawal. Future studies in rodents with N channel antagonists could help delineate the role of N-type channels in behavioral responses to chronic ethanol exposure.

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**Fig. 5.** Ethanol-induced increases in  $^{125}I$ - $\omega$ -conotoxin GVIA binding are reduced by a PKC inhibitor and by expression of the  $\epsilon$ V1 peptide. A, binding was measured in control (Con) cells, and in cells treated for 6 days with 1  $\mu$ M bisindolylmaleimide I (Bis), 120 mM ethanol (Eth), or ethanol + bisindolylmaleimide I (Eth + Bis). Data are means  $\pm$  S.E. ( $n = 5-9$ ) and are expressed as femtomoles per milligram cell protein. Means were compared by ANOVA and Scheffé  $F$  test. B, binding was measured in PC12 cells, vector-transfected cells (C2), and cells expressing the  $\delta$ V1 fragment (V182 or V183) or the  $\epsilon$ V1 fragment (V1e1 or V1e2). Data are means  $\pm$  S.E. ( $n = 6$ ) and are expressed as the percentage above or below binding measured in parallel control cells cultured without ethanol. \* $P < .01$  compared with PC12 or C cells (ANOVA and Newman-Keuls test).

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