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BIOCHEMICAL CHARACTERIZATION OF ETHANOL ACTIONS ON DIHYDROPYRIDINE-SENSITIVE CALCIUM CHANNELS IN BRAIN SYNAPTOSOMES

ALEX CANDA, BYUNG H. YU and PAUL Y. SZE*

Department of Pharmacology and Molecular Biology, The Chicago Medical School, North Chicago, IL 60064-3095, U.S.A.

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Abstract—This study was undertaken to investigate the biochemical events underlying the inhibitory action of ethanol on dihydropyridine-sensitive voltage-dependent Ca^{2+} channels in brain synaptosomes. The binding of radiolabeled dihydropyridine was used to determine functional Ca^{2+} channels in synaptosomes following exposure to ethanol. No effect on [^{3}H]PN 200-110 binding was found when disrupted synaptosomal membranes were incubated with ethanol concentrations as high as 300 mM, suggesting that ethanol did not interact directly with sites on or near the Ca^{2+} channels. However, when intact synaptosomes were first incubated with ethanol (100 mM) at 37° and then disrupted, a significant reduction in membrane binding of [^{3}H]PN 200-110 was found. Ethanol incubation of synaptosomes at 0° was ineffective. It appears that metabolic processes involving intracellular factors were required in the ethanol action. In examining this possibility, [^{3}H]PN 200-110 binding was activated by incubation of disrupted membranes with MgATP and Ca^{2+}-calmodulin, and ethanol was found to inhibit the activation in a concentration-dependent manner (50–200 mM). [^{3}H]PN 200-110 binding to membranes was also activated by incubation with MgATP and cyclic AMP-dependent protein kinase, but this activation was not inhibited by ethanol. These findings are consistent with the interpretation that ethanol acts on Ca^{2+} channels by inhibiting calmodulin-dependent activation of the channels.

Key words: ethanol; alcohol tolerance; alcoholism; calcium channels; dihydropyridine binding; brain synaptosomes

There has been substantial evidence that dihydropyridine-sensitive voltage-dependent Ca^{2+} channels are among the cellular targets of ethanol actions in the CNS [1]. The inhibition of depolarization-dependent Ca^{2+} influx by ethanol has been studied in detail in brain synaptosomes, suggesting that this action may be involved in the central depressant effect of ethanol [2–6]. The inhibition of Ca^{2+} influx by ethanol has also been shown in PC12 cells, a clonal neural cell line [7–9]. In animals chronically treated with ethanol, an increase of dihydropyridine binding sites has been found in brain membrane preparations, indicating that prolonged inhibition by ethanol leads to an up-regulation of the Ca^{2+} channels [10–12]. Similar adaptive increases of dihydropyridine binding sites following chronic exposure to ethanol have been demonstrated in PC12 cells [7, 13] and in cultured adrenal chromaffin cells as a neuronal model [14–16]. The up-regulation of the Ca^{2+} channels may be among the cellular mechanisms underlying the development of tolerance and dependence after chronic exposure to ethanol. This notion is supported by the findings that Ca^{2+} channel antagonists administered during withdrawal protect against withdrawal syndrome in animals [17–19] and humans [20]. Moreover, coadministration of Ca^{2+} channel antagonists during chronic channel exposure delays the acquisition of tolerance [21] and attenuates the signs of withdrawal excitability while concomitantly preventing the up-regulation of dihydropyridine binding sites [11, 12, 22].

Despite extensive research on the role of voltage-dependent Ca^{2+} channels in ethanol actions, the molecular mechanism whereby ethanol produces the inhibition of the channel activity remains unclear. In earlier studies, dihydropyridine binding in brain membrane preparations was not affected by ethanol *in vitro*, even at concentrations far exceeding those required to inhibit depolarization-dependent Ca^{2+} influx in synaptosomes [6, 23]. Therefore, it appears that ethanol does not interact directly with sites on or near the Ca^{2+} channels in altering their functional activity. The present study was undertaken to investigate the biochemical events that underlie ethanol inhibition of Ca^{2+} channel activity in brain synaptosomes.

MATERIALS AND METHODS

Animals and materials

Male Sprague–Dawley rats were supplied by a commercial breeder (Sasco King Animal Laboratories, Oregon, WI). (+)-[5-*Methyl*-^{3}H]PN 200-110 (70 Ci/mmol) was obtained from NEN Research Products (Boston, MA). Nifedipine, calmodulin (bovine brain), PKA† and its catalytic subunit (bovine heart), ATP, cAMP, PMSF and other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

Chronic ethanol administration

For chronic ethanol treatment, the rats were given a liquid diet (Liquidiet RAT L/D '82, BioServ, Inc.,

* Corresponding author. Tel. (708) 578-3000, Ext. 591 or 271; FAX (708) 578-3268.

† Abbreviations: PKA, protein kinase A; cAMP, cyclic AMP; PMSF, phenylmethylsulfonyl fluoride; GABA, γ-aminobutyric acid; and NMDA, *N*-methyl-D-aspartate.

Frenchtown, NJ) containing 6.0% (w/v) ethanol *ad lib.* for 3 weeks, as described in our previous studies [24–26]. This diet is based on the Lieber–DeCarli formulation [27]. For control of the liquid diet, one group of animals were given a control liquid diet, without ethanol but containing instead an isocaloric amount of maltose-dextrin, pair fed to the volume consumed by the ethanol-treated animals. Other control animals received standard laboratory food (Purina rat feed, PMI Feeds, St. Louis, MO) and water *ad lib.* Daily ethanol consumption was determined as follows: 9.3 ± 0.9 g/kg on day 7 of treatment; 13.5 ± 0.9 g/kg on day 14; and 14.2 ± 0.8 g/kg on day 20. Blood ethanol concentrations were 344 ± 75 mg/dL at the time of brain dissection (day 21). Body weight was 148 ± 11 g (day 1) and 195 ± 15 g (day 21) for the ethanol-treated group, and 140 ± 9 g (day 1) and 204 ± 13 g (day 21) for the pair-fed group (no significant difference in body weight gain between the two groups). The control animals fed laboratory food *ad lib.* were selected for matched body weight (210 ± 18 g) at the time of brain dissection. All values indicated above are means ± SEM (N = 10).

Preparation of synaptosomes

All animals were decapitated at 9:00–10:00 a.m., and the cerebral cortex was homogenized in 5 vol. of 0.32 M sucrose–10 mM Tris-HCl, pH 7.4. Synaptosomes were prepared as previously described [28]. Nuclei and cellular debris were pelleted by centrifuging at 1000 *g* for 10 min. The synaptosomes remaining in the supernatant were pelleted by a subsequent centrifugation at 10,000 *g* for 20 min. The pellet was resuspended in the homogenization buffer and placed on a discontinuous gradient of 0.85, 1.0, and 1.2 M sucrose. After centrifuging at 90,000 *g* for 90 min, the synaptosomal fraction was removed from the interface between the 1.0 and 1.2 M sucrose. The fraction was diluted with 4 vol. of 0.25 M sucrose–10 mM Tris-HCl, pH 7.4, and centrifuged at 15,000 *g* for 20 min to obtain the synaptosomal pellet.

Ethanol treatments in vitro

For experiments where intact synaptosomes were used, the synaptosomes were suspended in an isotonic buffer (20 mM Tris–HCl, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.4 mM NaH_2PO_4, 10 mM glucose, and 2 mM dithiothreitol) to give a protein concentration of 2 mg/mL. The suspended synaptosomes, 0.2 mL, were equilibrated with ethanol at 37° for 10 min. The synaptosomes were then disrupted by a Polytron (Brinkmann Instruments, Westbury, NY), and the membranes were pelleted at 48,000 *g* for 10 min for the determination of [^{3}H]PN 200-110 binding.

Incubation of synaptosomal membranes with compounds that promote phosphorylation of Ca^{2+} channels was carried out by modifications of a procedure described in a previous study [29]. After equilibration of the membranes with ethanol (10 min at 37°), the reaction by the calmodulin-dependent process was carried out in the presence of 50 mM Tris–HCl, pH 7.4, 10 mM $MgSO_4$, 0.5 mM ATP, 0.5 mM $CaCl_2$, 0.1 μM calmodulin and 0.05% β-mercaptoethanol. The reaction by the cAMP-dependent process was carried out in the presence of 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 0.5 mM ATP, 1 mM cAMP and 10 units/mL PKA. In both cases, the incubation (0.3 mg membrane protein/mL) was allowed to proceed at 37° for 1–5 min (as specified) and stopped by adding 4 vol. of ice-cold stop buffer (40 mM Na_2HPO_4, 10 mM KH_2PO_4, 50 mM NaF, 20 mM EDTA, 1 mM iodoacetamide and 0.1 mM PMSF). The membranes were then pelleted at 48,000 *g* for 10 min for the determination of [^{3}H]PN 200-110 binding.

Assay for dihydropyridine binding

High-affinity binding of dihydropyridine to synaptosomal membranes was determined by the procedure described previously in our laboratory [28], using [^{3}H]PN 200-110 as the ligand. The membranes (40–60 μg) from disrupted synaptosomes were incubated with [^{3}H]PN 200-110 (0.5 nM) in 200 μL of binding buffer (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 20 mM NaF, 2 mM dithiothreitol, and 0.1 mM PMSF). The binding was allowed to reach equilibrium at 22° (90 min in the dark). The membranes were separated by filtration on a Whatman GF/C filter and washed twice with 3 mL of ice-cold binding buffer. Radioactivity bound to the membranes was determined. Nonspecific binding, determined by 0.5 μM nifedipine, was less than 10% of total binding in all cases. For Scatchard analysis, the one-site model was used and linear regression analysis was applied to the data. For assessment of the difference between values, Dunnett's test for multiple comparisons or two-day ANOVA was used, as indicated under the results.

RESULTS

Ethanol effects in intact synaptosomes

As an initial step, the effect of ethanol on [^{3}H]PN 200-110 binding was examined in membranes from disrupted synaptosomes. The membranes were preincubated with ethanol (100 mM) at 37° for 10 min, and [^{3}H]PN 200-110 binding was determined in the ligand concentration range of 0.02 to 1 nM. As shown in the Scatchard analysis in Fig. 1, ethanol preincubation of the disrupted membranes produced no effect on [^{3}H]PN 200-110 binding. This lack of an effect by ethanol is in agreement with previous findings that ethanol as high as 600 mM did not alter [^{3}H]nitrendipine binding in membrane preparations [6, 23]. For comparison, disrupted synaptosomal membranes from animals with chronic treatment of ethanol were similarly examined for [^{3}H]PN 200-110 binding (Fig. 1). Scatchard analysis gave a B_{max} of 430 fmol/mg, as compared with a B_{max} of 268 fmol/mg for control animals. The significantly higher value of B_{max}, without a change in K_d for the ligand, represents the up-regulation of dihydropyridine binding sites as the result of chronic exposure to ethanol [10–12]. As in control membranes, [^{3}H]PN 200-110 binding in these membranes was not affected by ethanol at 100 mM. Higher ethanol concentrations up to 300 mM were similarly ineffective (not shown). It should be emphasized that for control of the liquid diet effect in the chronic ethanol treatment, the membranes from animals pair-fed with a control liquid diet were also analyzed for [^{3}H]PN 200-110 binding in the absence and presence of ethanol. These membranes yielded identical results (not shown) as the membranes from control animals receiving standard laboratory food (shown in Fig. 1). There-

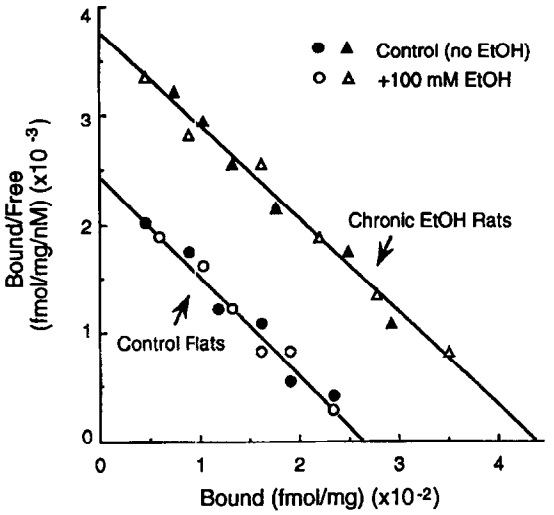

Fig. 1. Scatchard analysis of [^{3}H]PN 200-110 binding in disrupted synaptosomal membranes preincubated with ethanol. Ligand binding was determined after the membranes were preincubated with ethanol (EtOH). Each point is the mean from 6 determinations. Linear regression analysis was applied to the data. For control rats (receiving standard laboratory food *ad lib.*): control condition (●): r (correlation coefficient) = 0.987; K_d = 0.110 ± 0.011 nM; B_{max} = 268 ± 12 fmol/mg; at 100 mM ethanol (○): r = 0.985; K_d = 0.116 ± 0.009 nM; B_{max} = 275 ± 10 fmol/mg. These two lines were not significantly different (only the control line is shown). For chronic EtOH rats (chronically treated with ethanol): control condition (▲): r = 0.990; K_d = 0.107 ± 0.011 nM; B_{max} = 430 ± 17 fmol/mg (significantly different from control B_{max}, P < 0.001); at 100 mM ethanol (△): r = 0.992; K_d = 0.110 ± 0.010 nM; B_{max} = 445 ± 19 fmol/mg. These two lines were not significantly different (only the control line is shown).

fore, the dietary difference did not pose as a variable to dihydropyridine binding in the membranes.

When intact synaptosomes were preincubated with ethanol (100 mM) at 37° for 10 min and then disrupted for the determination of [^{3}H]PN 200-110 binding, the binding was found to be reduced markedly (Fig. 2A). Scatchard analysis shows that the inhibition of [^{3}H]PN 200-110 binding was due to a decrease in B_{max} (158 fmol/mg as compared with 255 fmol/mg under the control condition) without a significant change in K_d for the ligand. Thus, in intact synaptosomes, ethanol preincubation produced a reduction in the number of active dihydropyridine binding sites. In a parallel experiment, intact synaptosomes were preincubated with ethanol at 0° rather than 37°. No change in [^{3}H]PN 200-110 binding was found even after extended preincubation such as 30 min (data not shown). Thus, preincubation at 37° was a necessary condition for the ethanol action to occur.

For comparison, synaptosomes from animals chronically exposed to ethanol were similarly examined for ethanol inhibition of [^{3}H]PN 200-110 binding (Fig. 2B). At 100 mM ethanol, the inhibition was marginal and insignificant. When ethanol concentration was increased to 200 mM, a significant inhibition of binding was found, with the B_{max} reduced to 267 fmol/mg from the control value of 418 fmol/mg. It appears that chronic ethanol exposure resulted in the resistance of dihydropyridine binding to the inhibitory action of ethanol *in vitro*.

To assess this resistance to ethanol inhibition in a quantitative manner, concentration–response relationships were compared in synaptosomes from control and chronically ethanol-treated animals. After preincubation

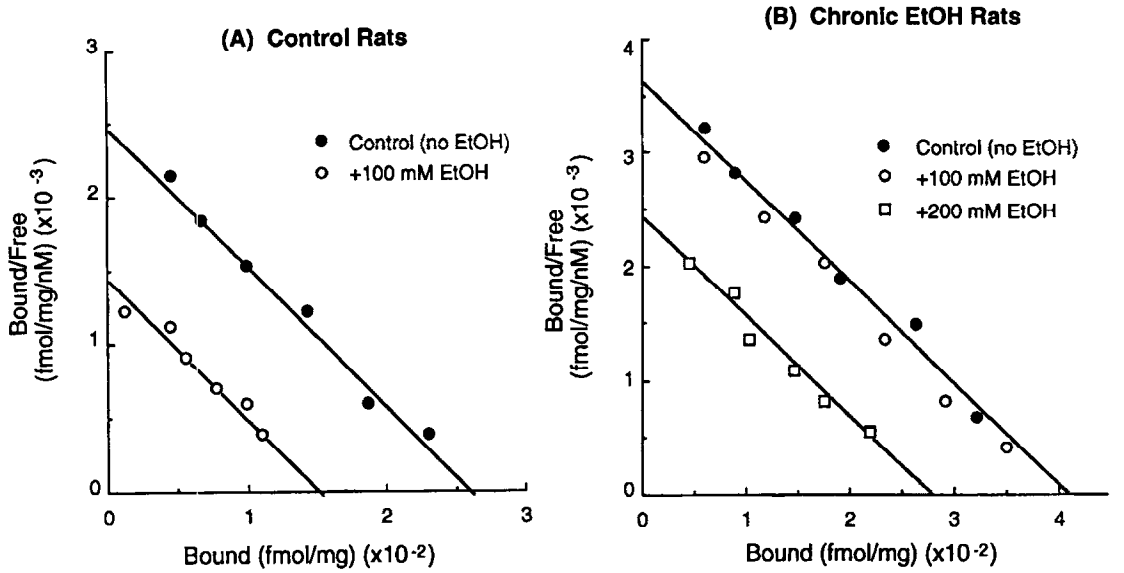

Fig. 2. Scatchard analysis of [^{3}H]PN 200-110 binding after ethanol preincubation of intact synaptosomes. Ligand binding was determined in membranes after intact synaptosomes were preincubated with ethanol and then disrupted. Each point is the mean from 6 determinations. (A) Control rats (receiving standard laboratory food *ad lib.*). Control condition (●): r = 0.994; K_d = 0.115 ± 0.008 nM; B_{max} = 255 ± 11 fmol/mg; at 100 mM ethanol (○): r = 0.991; K_d = 0.120 ± 0.008 nM; B_{max} = 158 ± 9 fmol/mg (significantly different from control B_{max}, P < 0.001). (B) Chronic EtOH rats (chronically treated with ethanol). Control condition (●): r = 0.988; K_d = 0.108 ± 0.009 nM; B_{max} = 418 ± 15 fmol/mg (significantly different from control B_{max} shown in panel A, P < 0.001); at 100 mM ethanol (○): r = 0.990; K_d = 0.105 ± 0.010 nM; B_{max} = 390 ± 14 fmol/mg. These two lines were not significantly different (only the control line is shown). At 200 mM ethanol (□): r = 0.992; K_d = 0.111 ± 0.012 nM; B_{max} = 267 ± 11 fmol/mg (significantly different from control B_{max}, P < 0.001).

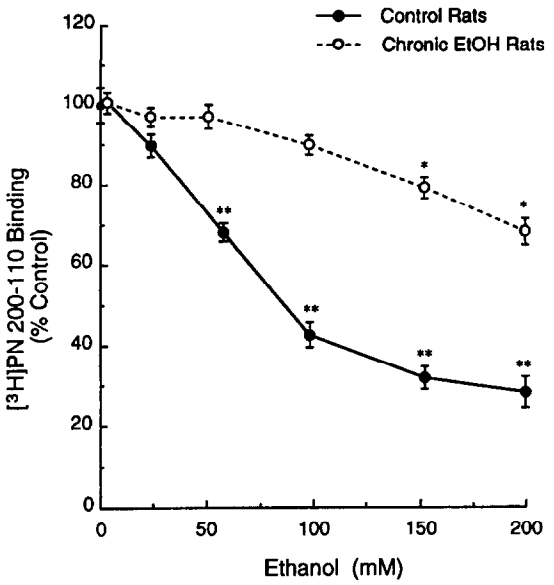

Fig. 3. Concentration effects of ethanol on [^{3}H]PN 200-110 binding after ethanol preincubation of intact synaptosomes. The animals and preincubation procedure were as described in the legend of Fig. 2, and equilibrium binding was determined at 0.5 nM [^{3}H]PN 200-110. At the ligand concentration used, the value at zero ethanol for control rats was 195 ± 12 fmol/mg, and for chronic EtOH rats, 305 ± 12 fmol/mg. Both were normalized to 100% to facilitate comparison. Each value is the mean ± SEM from 4 determinations. Significant difference from zero ethanol control (Dunnett's test): (*) $P < 0.05$; and (**) $P < 0.01$.

of intact synaptosomes with ethanol (25–200 mM), the disrupted membranes were examined for [^{3}H]PN 200-110 binding (Fig. 3). In membranes from control animals, ethanol at 25 mM produced a threshold and insignificant inhibition (10%), but as ethanol reached 50 mM, the inhibition (32%) was significant. At 100 and 200 mM ethanol, the inhibition was 58 and 70%, respectively. In membranes from animals receiving chronic ethanol treatment, ethanol at 100 mM was without a significant effect on [^{3}H]PN 200-110 binding. Only when ethanol concentration reached 150 mM did an inhibition of binding become significant (22%). At 200 mM ethanol, the inhibition of binding was 31%. Thus, the inhibition of [^{3}H]PN 200-110 binding at 200 mM ethanol was comparable to that found at 50 mM ethanol in control membranes. These results indicate that under the incubation conditions *in vitro*, dihydropyridine binding sites from animals with chronic ethanol exposure were approximately 4 times less sensitive to ethanol inhibition than those from control animals.

Ethanol effects on calmodulin-dependent activation of [^{3}H]PN 200-110 binding

Since preincubation of intact synaptosomes with ethanol at 37° was a necessary condition for the inhibition of [^{3}H]PN 200-110 binding to occur, it appears that metabolic reactions requiring intracellular factors are involved in the inhibitory action of ethanol. To examine this possibility, attempts were made to modulate dihydropyridine binding by endogenous factors that are known to phosphorylate Ca^{2+} channels. In this experiment, synaptosomal membranes were incubated at 37° in the presence of 0.5 mM ATP, 10 mM Mg^{2+}, 0.5 mM Ca^{2+}, and 0.1 μM calmodulin ("calmodulin system"). The membranes were then pelleted for the determination of [^{3}H]PN 200-110 binding. As shown in the time course in Fig. 4, the activation of [^{3}H]PN 200-110 binding by the calmodulin system was rapid, reaching a near plateau at 5 min. At 5 min, the increase of binding was 165% above the basal value. It should be mentioned that the concentration of calmodulin used (0.1 μM) produced optimal ethanol inhibition of binding under the incubation conditions. The requirement for the four components (ATP, Mg^{2+}, Ca^{2+} and calmodulin) in the activation is summarized in Table 1. MgATP alone or Ca^{2+}-calmodulin alone was not sufficient to produce the activation. The addition of the Ca^{2+}-chelator EGTA abolished the effect of the calmodulin system. In Scatchard analysis, the activation resulted in the increase of B_{max} without a significant change in K_d (not shown), indicating the increase of the number of active dihydropyridine binding sites. In membranes preincubated with ethanol, the activation of [^{3}H]PN 200-110 binding was inhibited markedly (Fig. 4). At 100 mM ethanol, the increase of binding after a 5-min incubation was only 70% above the basal value.

The concentration–response relationship of the effect of ethanol on the activation was determined for ethanol concentrations of 25–200 mM (Fig. 5). In the absence of ethanol, the increase of [^{3}H]PN 200-110 binding after a 5-min incubation with the calmodulin system was 175% above the basal value. In the presence of 25 mM ethanol, only a threshold and insignificant decrease of the activation was shown. At 50 mM ethanol, the reduction of the activation was small but significant. When ethanol reached 100 mM, the activation was only 75% above the basal value, an inhibition of 58% (when compared with the 175% activation in the absence of ethanol). At 200 mM ethanol, the activation was reduced further to only 37% above the basal value, an inhibition as much as 83%. These data indicate that ethanol inhibits the activation of [^{3}H]PN 200-110 binding in a concentration-dependent manner. It is interesting to note that the profile of the concentration–response relationship obtained here resembles that of the inhibition of [^{3}H]PN 200-110 binding by ethanol preincubation of intact synaptosomes (see Fig. 3).

Table 1. Requirement in the calmodulin-dependent activation of [^{3}H]PN 200-110 binding

Addition	[^{3}H]PN 200-110 binding (% of control)
None (control)	100 ± 4.5
ATP	99 ± 5.0
Ca^{2+} + CaM	105 ± 5.2
ATP + Ca^{2+} + CaM	298 ± 14.0*
ATP + Ca^{2+} + CaM + EGTA	95 ± 3.2

The basal buffer contained 10 mM Mg^{2+}. Concentration of the additions: ATP, 0.5 mM; $CaCl_2$, 1 mM; calmodulin (CaM), 0.1 μM; and EGTA, 2 mM. After incubation (5 min, 37°), equilibrium binding was determined at 0.5 nM [^{3}H]PN 200-110 as described in the text. The control value was 210 ± 9.5 fmol/mg (100%). Each value is the mean ± SEM from 4 determinations.

* Significant difference from control (Dunnett's test): $P < 0.01$.

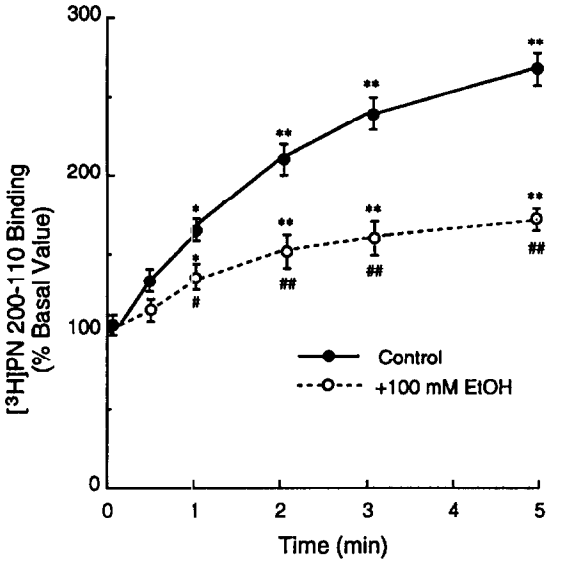

Fig. 4. Time course of the activation of [^{3}H]PN 200-110 binding in synaptosomal membranes by the calmodulin-dependent process. The membranes were preincubated in the presence and absence of 100 mM ethanol (37°, 10 min) and then incubated with the following: 0.5 mM ATP, 10 mM Mg^{2+}, 1 mM Ca^{2+} and 0.1 μM calmodulin (see text for details). The membranes were pelleted and equilibrium binding was determined at 0.5 nM [^{3}H]PN 200-110. The basal value (zero time) was 182 ± 10 fmol/mg (100%). Each value is the mean ± SEM from 5–7 determinations. Significant difference (two-way ANOVA) from zero time value: (*) $P < 0.01$; and (**) $P < 0.005$. Significant difference from the corresponding control (in the absence of ethanol): (#) $P < 0.01$; and (##) $P < 0.005$.

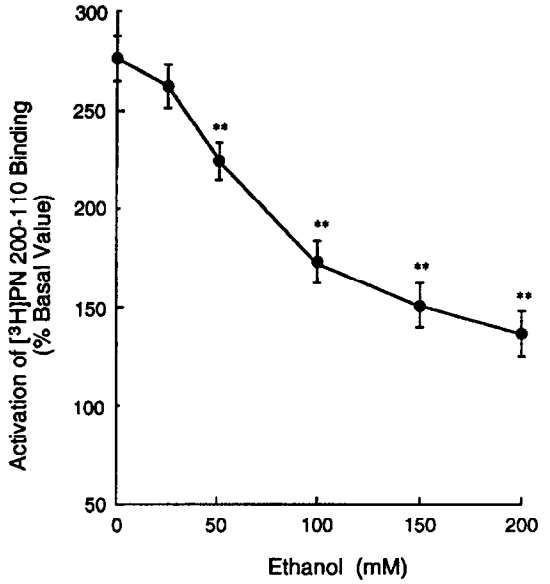

Fig. 5. Concentration effects of ethanol on calmodulin-dependent activation of [^{3}H]PN 200-110 binding in synaptosomal membranes. The experimental conditions were as described in the legend of Fig. 4, and the incubation time was 5 min. The activation at zero ethanol was 255 ± 16% of basal value (the value before activation, see Fig. 4). Each value is the mean ± SEM from 4 determinations. Significant difference from the zero ethanol control (Dunnett's test): (**) $P < 0.01$.

Ethanol effects on cAMP-dependent activation of [^{3}H]*PN 200-110 binding*

In another experiment, [^{3}H]PN 200-110 binding to synaptosomal membranes was also found to be activated by incubation with 0.5 mM ATP, 10 mM Mg^{2+}, 1 mM cAMP and 10 units/ mL PKA ("cAMP system"). The time course of this activation, shown in Fig. 6, was similar to that produced by the calmodulin system. At 5 min after the incubation, the increase of [^{3}H]PN 200-110 binding was almost 200% above the basal value. The use of PKA at 10 units/mL concentration produced the maximal activation of the binding under our incubation conditions. The requirement for the four components (ATP, Mg^{2+}, cAMP and PKA) in the activation is summarized in Table 2. No activation occurred when cAMP or PKA was absent from the incubation. The activation was fully produced when the catalytic subunit of PKA was substituted for cAMP and PKA. Thus, the addition of exogenous PKA or its catalytic subunit was an absolute requirement for the activation to occur. It is possible that certain endogenous membrane-bound factors, such as PKA, are labile and could be lost during membrane isolation. In electrophysiological analysis, the addition of exogenous PKA and MgATP has been shown to prevent the rapid rundown of Ca^{2+} channel activity recorded from cell-free membrane patches [30]. Of particular note here is the effect of ethanol on the activation (Fig. 6). In contrast to the inhibition found in the calmodulin-dependent activation, ethanol at 100 mM did not affect the activation of [^{3}H]PN 200-110 binding by the cAMP-dependent process. No effect was found even at ethanol concentrations as high as 300 mM (not shown).

DISCUSSION

The primary objective of these experiments was to characterize the biochemical action of ethanol on voltage-dependent Ca^{2+} channels. There are two relevant points to be made in this regard. First, ethanol does not appear to inhibit Ca^{2+} channels by direct biochemical interaction with sites on or near the channels, thus altering their functional state. No change in dihydropyridine

Table 2. Requirement in the cAMP-dependent activation of [^{3}H]PN 200-110 binding

Addition	[^{3}H]PN 200-110 binding (% of control)
None (control)	100 ± 4.2
ATP	98 ± 5.0
ATP + cAMP	105 ± 6.3
PKA + cAMP	94 ± 3.8
ATP + cAMP + PKA	310 ± 12.8*
ATP + CS/PKA	286 ± 11.7*

The basal buffer contained 10 mM Mg^{2+}. Concentration of the additions: ATP, 0.5 mM; cAMP, 1 mM; PKA or the catalytic subunit (CS/PKA), 10 units/mL. After incubation (5 min, 37°), equilibrium binding was determined at 0.5 nM [^{3}H]PN 200-110. The control value was 190 ± 8.0 fmol/mg (100%). Each value is the mean ± SEM from 4 determinations.

* Significant difference from control (Dunnett's test): $P < 0.01$.

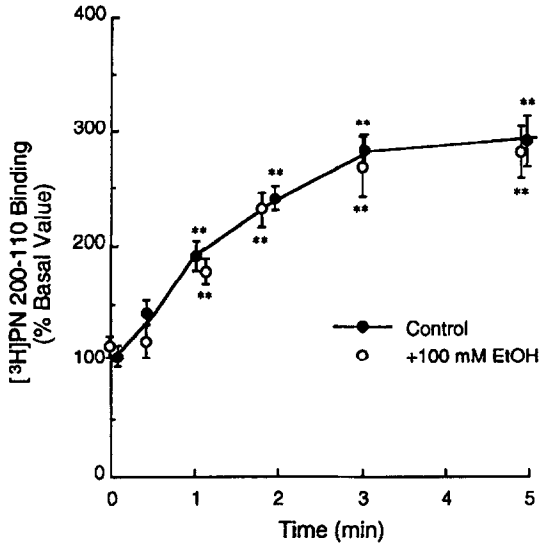

Fig. 6. Time course of the activation of [^{3}H]PN 200-110 binding in synaptosomal membranes by the cAMP-dependent process. The experimental procedure was the same as in Fig. 4, except that the following incubation conditions were used: 0.5 mM ATP, 10 mM Mg^{2+}, 1 mM cAMP and 10 units/mL PKA (see text for details). The basal value was 205 ± 11 fmol/mg (100%). Each value is the mean ± SEM from 5–7 determinations. Significant difference (two-way ANOVA) from zero time value: (**) $P < 0.005$. No significant difference was found between all values in the absence (control) and presence of 100 mM ethanol; therefore, only the control line is shown.

binding occurred when isolated membranes were exposed to ethanol, as shown here as well in previous studies [6, 23]. It is generally agreed that in biochemical analysis of dihydropyridine-sensitive voltage-dependent Ca^{2+} channels in isolated (depolarized) membrane preparations, there is a correlation between the number of high-affinity dihydropyridine binding sites and the number of functional Ca^{2+} channels (active channels that can open) [31]. Thus, in isolated synaptosomal membranes exposed to ethanol, the alteration of the functional state of Ca^{2+} channels was not evident from the results on dihydropyridine binding. Instead, when intact synaptosomes were preincubated with ethanol at 37° and then disrupted for the determination of [^{3}H]PN 200-110 binding, a significant reduction of the dihydropyridine binding sites occurred. Preincubation with ethanol at 0° was ineffective. The fact that preincubation in whole synaptosomes at 37° was a necessary condition to produce the inhibition suggests that metabolic reactions requiring intracellular factors were involved.

Second, in examining the possibility that ethanol inhibits metabolic reactions that modulate Ca^{2+} channel activity, we showed that dihydropyridine-sensitive Ca^{2+} channels in isolated synaptosomal membranes were activated by the addition of MgATP and Ca^{2+}-calmodulin, and that ethanol inhibited this activation in a concentration-dependent manner. In cell-free membrane patches (i.e. isolated membranes), it has been demonstrated that voltage-dependent Ca^{2+} channels must be phosphorylated in order to open when the membranes are depolarized [30]. Dihydropyridine-sensitive Ca^{2+} channels in the membrane-bound state can be phosphorylated *in vitro* by calmodulin-dependent and cAMP-dependent protein kinases as well as by protein kinase C [29]. While all the three modes of phosphorylation have been implicated in the regulation of neuronal voltage-dependent Ca^{2+} channels [30, 32–34], direct evidence is still lacking and the precise operation of phosphorylation by specific protein kinases in neuronal cells *in vivo* remains to be understood. If, however, calmodulin-dependent phosphorylation does operate *in vivo* in the regulation of voltage-dependent Ca^{2+} channels in synaptosomes (synaptic terminals), the inhibition of this process by ethanol would be important. In this scenario, functional (i.e. phosphorylated) Ca^{2+} channels undergo rapid turnover of dephosphorylation and phosphorylation, and the inhibition of phosphorylation by ethanol results in a rapid reduction in the number of functional channels. The data on the reduction of [^{3}H]PN 200-110 binding following ethanol preincubation of intact synaptosomes are compatible with such a notion. We further showed that [^{3}H]PN 200-110 binding in synaptosomal membranes was also activated by the cAMP-dependent process, which required the addition of an exogenous protein kinase under our experimental conditions. This cAMP-dependent activation of [^{3}H]PN 200-110 binding was not affected by ethanol. Although the effect of ethanol on the possible activation by the protein kinase C process remains to be determined, we clearly demonstrated a selectivity in the inhibitory action of ethanol between the two processes examined. The selective inhibition of the calmodulin-dependent process may be related to the action of ethanol on membrane binding of calmodulin (see below).

Using patch clamp techniques, recent studies have examined the effects of ethanol on Ca^{2+} currents in whole nerve terminals (synaptosomes) from rate neurohypophysis [35, 36]. The results show that long-lasting Ca^{2+} currents, which are generated by "L-type" voltage-dependent Ca^{2+} channels (sensitive to dihydropyridine), can be inhibited by ethanol as low as 10–25 mM. In our biochemical analysis of [^{3}H]PN 200-110 binding, only a small and statistically insignificant inhibition was detected at ethanol concentrations of 25 mM. A similar requirement for ethanol concentrations higher than 25 mM was reported in earlier studies on the inhibition of depolarization-induced $^{45}Ca^{2+}$ uptake in brain synaptosomes [2–4] and in cultured PC12 cells [7]. It is possible that electrophysiological techniques are more sensitive than biochemical approaches in detecting small changes of Ca^{2+} channel activities. It is also possible that Ca^{2+} channels in neurohypophysis are more susceptible to inhibition by ethanol than those in the cerebral cortex examined in the biochemical studies on synaptosomes. In research on ethanol actions in the CNS, it is generally thought that neuronal alterations found at low concentrations of ethanol *in vitro* (<25 mM) are relevant to CNS responses to moderate ethanol intake *in vivo* (such as the alteration of $GABA_A$ receptors and NMDA receptors), whereas neuronal changes produced by higher concentrations of ethanol (>25 mM) may be relevant *in vivo* only under heavy ethanol intoxication conditions [37]. According to this notion and taking into consideration only the biochemical data, the results from [^{3}H]PN 200-110 binding in this study and from depolarization-induced $^{45}Ca^{2+}$ uptake in earlier studies would indicate that voltage-dependent Ca^{2+} channels in the CNS are involved only in response to high doses of ethanol.

An additional aspect of our results is the demonstration that the resistance of Ca^{2+} channels to acute ethanol

inhibition, occurring after chronic ethanol exposure, was reflected in [^{3}H]PN 200-110 binding. By examining [^{3}H]PN 200-110 binding in synaptosomes from animals chronically exposed to ethanol, we showed that the concentration–response curve of ethanol inhibition of the dihydropyridine binding was shifted, and higher concentrations of ethanol were required to produce the inhibition of binding in these synaptosomes than in control synaptosomes. These data support the earlier finding that in depolarization-induced synaptosomal Ca^{2+} uptake, resistance to ethanol inhibition occurred after chronic ethanol exposure [2–4]. Thus, in addition to the up-regulation of Ca^{2+} channels, which appears to involve gene expression for *de novo* synthesis of channel proteins [15], adaptive responses to chronic ethanol exposure also include the resistance of the Ca^{2+} channels to the acute inhibitory action of ethanol.

The selective inhibition of the calmodulin-dependent activation of Ca^{2+} channels, shown in our study, poses an interesting question as to the mechanism of the ethanol action. We have reported recently that ethanol inhibits calmodulin binding to synaptosomal plasma membranes from rat brain and that in animals chronically exposed to ethanol, the membranes are resistant to the inhibitory action of ethanol on calmodulin binding [24, 26, 38]. Other studies have shown that calmodulin antagonists, such as trifluoperazine, calmidazolium and W-7, inhibit depolarization-dependent $^{45}Ca^{2+}$ influx in PC12 cells [34] and adrenal medullary cells [39]. Moreover, [^{3}H]nitrendipine binding in brain membrane preparations is inhibited by calmodulin antagonists [40]. Therefore, we postulate that ethanol inhibition of calmodulin binding to the membranes is related to the inhibition of calmodulin-dependent activation of Ca^{2+} channels. The resistance of dihydropyridine binding sites to ethanol inhibition, found after chronic ethanol exposure, may similarly be attributed to the resistance of the membranes to ethanol inhibition of calmodulin binding. This working hypothesis of a calmodulin-mediated action may guide future studies.

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