Increase in Expression of $\alpha_1$ and $\alpha_2/\delta_1$ Subunits of L-Type High Voltage-Gated Calcium Channels After Sustained Ethanol Exposure in Cerebral Cortical Neurons

Masashi Katsura$^1$, Masahiro Shibasaki$^1$, Shinsuke Hayashida$^1$, Fumiko Torigoe$^1$, Atsushi Tsujimura$^2$, and Seitaro Ohkuma$^1$*

$^1$Department of Pharmacology, Kawasaki Medical University, 577 Matsushima, Kurashiki 701-0192, Japan
$^2$Department of Biochemistry and Molecular Genetics, Research Institute for Neurological Diseases and Geriatrics, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamikyo-ku, Kyoto 602-8401, Japan

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Abstract. Previous reports revealed up-regulation of L-type high voltage-gated calcium channels (HVCCs) in mouse brains with ethanol physical dependence. We investigated mechanisms of enhancement of L-type HVCC function using mouse cerebrocortical neurons exposed to 50 mM ethanol for 3 days and the brains of mouse physically dependent on ethanol. Ethanol facilitated 30 mM KCl-stimulated $^{45}$Ca$^{2+}$ influx in dose- and duration-dependent manners, which was abolished by nifedipine, an inhibitor specific to L-type HVCCs, but not by inhibitors for other types of HVCCs. Increase in $[^3]$H]PN200-110 binding to the particulate fractions from the ethanol-treated neurons was due to increased $B_{\text{max}}$ value with no changes in $K_{d}$ value. Western blot analysis showed the increased expression of $\alpha_1$C, $\alpha_1$D, and $\alpha_2/\delta_1$ subunits with decreased $\beta_4$ subunit expression and no changes in expressions of $\alpha_1$A, $\alpha_1$B, $\alpha_1$F, and $\alpha_2$ subunits. A similar pattern of the changes in the expression of these subunits of L-type HVCCs were observed in the cerebral cortex from mouse with ethanol physical dependence. These results indicate that sustained ethanol exposure to the neurons induces up-regulation of L-type HVCCs, which is due to increased expressions of $\alpha_1$C, $\alpha_1$D, and $\alpha_2/\delta_1$ subunits, and produces no alterations in P/Q- and N-type HVCC functions.

Keywords: ethanol (alcohol) physical dependence, high voltage-gated calcium channel, Ca$^{2+}$ influx, $[^3]$H]PN200-110 binding, cerebral cortical neuron

Introduction

Despite a variety of investigations with neurochemical and pharmacological approaches on neurotransmitter turnover, neurotransmitter receptors and intracellular signal transduction systems coupling to neurotransmitter receptors to elucidate mechanisms of alcohol (ethanol) dependence and withdrawal syndrome, the previously reported data have been controversial (1, 2). Among supposed biochemical events involved in development of drug dependence, several studies have reported the increase in the binding of radiolabeled dihydropyridines to brain preparations and cultured cells with neuronal origin after chronic ethanol treatment (3–8), which suggests the increase in the number of L-type high voltage-gated calcium channels (HVCCs). Chronic treatment with ethanol also induces increases in inward Ca$^{2+}$ currents (9, 10) and Ca$^{2+}$ influx (4, 6, 11). From these data, it is reasonable to conclude that chronic exposure to ethanol produces increased Ca$^{2+}$ entry into neurons via L-type HVCCs and this event may cause alterations in neuronal functions participating in development of alcohol dependence.

Since the 1990’s, studies using molecular biological procedures have revealed that HVCCs are a heterometric complex composed of $\alpha_1$, $\alpha_2/\delta$, $\beta$, and $\gamma$ subunits. Among these subunits, $\alpha_1$ subunits form a pore through which Ca$^{2+}$ passes and other subunits play roles to
modulate α1 subunit functions (8, 12, 13). However, there are few available data on the mechanisms of functional increase in L-type HVCCs, that is, which L-type HVCC subunits are involved in the up-regulation of the HVCCs after chronic exposure to ethanol. We therefore examined the alterations of the expression of the subunits composing L-type HVCCs using primary cultures of mouse cerebral cortical neurons after sustained ethanol exposure and the cerebral cortex of mouse chronically administered ethanol.

Materials and Methods

Primary culture of mouse cerebral cortical neurons
The procedures to isolate and culture the neurons are described elsewhere in detail (14). In short, the neopallium from a 15-day-old fetus of ddY strain mouse was enzymatically digested and centrifuged. The resultant pellet was suspended in Dulbecco’s modified Eagle medium (DMEM) with 15% fetal bovine serum for 3 days in humidified 95% air/5% CO₂ at 37°C. After the incubation of the cells with 10 μM cytosine arabinoside for 24 h to suppress non-neuronal cells, the neurons were then cultured in DMEM supplemented with 10% of horse serum under the conditions described above. The percentage of the neurons in the cultured cells was more than 95% (15, 16). The culture medium was exchanged to fresh DMEM containing 10% horse serum every four days. After culturing for 10 days, the neurons were used in the following experiments.

The neurons used here have been already reported to possess, at least, four types of HVCCs such as P/Q-, N-, and L-type HVCCs (17, 18).

The stimulation of 30 mM KCl was previously reported to depolarize the neuronal membrane (18–20).

Preparation of alcohol-dependent mice
Alcohol-dependent mice were prepared according to the method previously reported with a minor modification (21, 22). Male ddY strain mice weighing 30 g purchased from Japan SLC, Inc. (Hamamatsu) were kept with laboratory chow (Oriental Yeast Co., Ltd., Chiba) and tap water ad libitum for one week under a 12-h light and 12-h dark cycle, and then the animals inhaled pyrazole (1.0 mmol/kg per day, once a day) to inhibit enzymatic degradation of ethanol. Mice receiving pyrazole alone were used as the control. With these procedures, all mice showed typical withdrawal signs such as tonic-clonic seizures with grimaces and head twitches (21), indicating that all mice were physically dependent on ethanol. Our previous report demonstrated that the blood alcohol concentrations immediately after the withdrawal from ethanol vapor were about 290 mg/dl of blood measured by the previously reported enzymatic method (9, 23).

The animals were used immediately after the last administration of ethanol vapor for immunoblot analysis of HVCC subunit expression.

These experiments using mice were approved by the Animal Research Committee of Kawasaki Medical School and conducted according to the “Guideline for the care and use of Laboratory Animals” of Kawasaki Medical University based on the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

Sustained exposure of the neurons to 50 mM ethanol
Ethanol diluted with Hank’s solution was directly added into the culture medium (24). At the first exposure of the neurons to ethanol, 5 M ethanol in Hank’s solution with 1/100 times volume of the culture medium was added into the culture medium, and then a half volume of 5 M ethanol solution was added into the medium every 12 h. The additional ethanol was poured into the medium at the same spot culture dish where the first addition of ethanol was carried out.

These procedures did not float the cells around the spot for addition of ethanol and maintained the ethanol concentration in the medium at 51.9 ± 7.7 mM (N = 59) during the 5-day period; the ethanol concentration was measured according to the previous method (25) using an enzymatic assay kit (TaKaRa FD kit; Takara, Tokyo). The ethanol (50 mM) exposure for three to five days showed no neurotoxicity, as assessed by measuring leakage of lactic dehydrogenase (LDH) activity from the neurons and activity of the neurons to exclude trypan blue dye (data not shown).

Measurement of 45Ca²⁺ influx
Influx of ⁴⁵Ca²⁺ into the neurons was measured according to the previous method (17, 18). In brief, after preincubation of the neurons in Ca²⁺-free Krebs-Ringer bicarbonate buffer (137 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·6H₂O, 25 mM NaHCO₃, and 10 mM glucose, pH 7.4) containing 20 mM Heps (KRB-H) at 37°C for 10 min, the neurons were incubated in fresh and warm (37°C) Ca²⁺-free KRB-H. The reaction was initiated by simultaneous addition of 30 mM KCl and 2.7 mM CaCl₂·H₂O (1.0 μCi of [⁴⁵Ca²⁺]Cl⁻/dish). After the incubation of the neurons at 37°C for 2 min, the radiolabeled Ca²⁺-containing incubation buffer was discarded and the neurons were washed 5 times with ice-cold KRB-H containing 2.7 mM CaCl₂·H₂O (total volume: 7.5 ml).
Thereafter, the neurons were scraped off from a culture dish with 0.5 M NaOH. An aliquot of the alkaline digested neurons was neutralized with equimolar acetic acid and then used to measure radioactivity accumulated in the neurons by liquid scintillation spectrometry. The $^{44}$Ca$^{2+}$ influx without any stimulation by 30 mM KCl was represented as the basal value.

Nifedipine, used for examining the properties of $^{44}$Ca$^{2+}$ influx changed after ethanol exposure, was dissolved in 0.01% DMSO and 100 times concentrated nifedipine solution was added into the medium. The final concentration of DMSO (0.0001%) alone did not show any effects on $^{44}$Ca$^{2+}$ influx (data not shown).

The concentrations of various inhibitors for HVCCs used here inhibited maximally the functions of HVCCs as reported previously (18).

**Measurement of $[^3H]$PN200-110 and $[^3H]$diltiazem bindings**

$[^3H]$PN200-110 binding to the neuronal particulate fractions was measured according to the previous methods (26, 27) with a minor modification. Proteins (300 µg) were incubated for 1 h at 25°C under dim red light illumination in 500 µl of PN200-110 binding buffer [50 mM Tris-HCl buffer (pH 7.4), 1.25 mM CaCl$_2$, 1.25 mM MgCl$_2$] with concentrations of $[^3H]$PN200-110 varying from 0.0625 to 2.5 nM. Non-specific binding was assessed in the presence of 10 µM unlabeled (±)PN200-110. Bound ligand was assessed by filtration through Whatman GF/B filters followed by three washes with 6 ml of the binding buffer.

$[^3H]$Diltiazem binding was carried out according to the previous method (28) with a minor modification. The particulate fractions prepared from the neurons were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mg/ml of bovine serum albumin (200 µg protein/500 µl of assay volume) and incubated with $[^3H]$diltiazem (0.125 – 32 nM) at 25°C for 120 min. The reaction was terminated by filtration of the reaction mixture through Whatman GF/B filters precoated with 0.3% polyethyleneimine at 4°C for 5 h, under vacuum followed by three times washes of the filters with ice-cold 50 mM Tris-HCl buffer. The filter was then used to measure the radioactivity retained on the filter by liquid scintillation spectrometry. Specific binding was calculated by subtraction of non-specific binding obtained in the presence of 10 µM non-labeled diltiazem from total binding determined without non-labeled diltiazem.

The particulate fractions used for $[^3H]$PN200-110 and $[^3H]$diltiazem bindings were prepared as described below. The neurons were washed three times with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$, pH 7.4), scraped off from the dishes with ice-cold 50 mM Tris-HCl buffer (pH 7.4), homogenized with a Polytron homogenizer, and centrifuged (48,000 × g, 4°C, 20 min). The washing procedure by centrifugation after homogenization was carried out 4 times before stockng at ~80°C for at least 24 h. Before the binding experiment, the frozen pellet was thawed, suspended with the same buffer used for preparing the particulate fractions, and washed three times as described above.

**Immunoblots for subunits of HVCCs**

Extraction of proteins from the neurons for electrophoresis was carried out as follows. The neurons were washed five times with ice-cold 0.15 M NaCl, fixed with 6% trichloracetic acid (TCA) in 0.15 M NaCl at 4°C, scraped off from the dishes, and centrifuged (10,000 × g, 5 min, 4°C). The pellet was washed with ice-cold Tris-HCl buffer (pH 7.4), mixed with the sample buffer [4% sodium lauryl sulfate (SDS), 12% β-mercaptoethanol, and 20% glycerol in 100 mM Tris-HCl (pH 6.8)], sonicated (1 min), boiled (3 min), and finally centrifuged (10,000 × g, 60 min, 4°C). The resultant supernatant was stored at ~80°C until use.

In order to prepare protein extract from the cerebral cortex of mouse, a Teflon cannula was inserted into the left ventricle of the mouse deeply anesthetized by intraperitoneal injection of sodium thiopental and the abdominal aorta was cut off. Thereafter, the brain was perfused with saline containing 0.15 M NaCl with the flow rate of 20 ml/min for 1 min and then perfused with 6% TCA in saline for 3 min with same flow rate described above. The cerebral cortex was dissected from the perfused brain, sonicated, and then centrifuged. The resultant pellet was treated with the same procedures to extract proteins from the neurons.

After electrophoresis (applied proteins, 20 µg/lane), gel size of 10 × 10 cm and thickness of 0.5 mm (20 mA, 90 min), proteins separated on the gel were transferred to a nitrocellulose filter with a semidy my type transblotter (160 mA, 120 min). The nitrocellulose filters were incubated with antibodies against each subunit of the HVCCs [diluted 1:200 to 1:1,000 in Tris-buffered saline (TBS; 0.15 M NaCl and 20 mM Tris-HCl, pH 7.4) containing 0.1% normal serum] after washes of the filters with PBS and subsequent blocking with TBS at room temperature for 60 min. After the filters were washed four times with TBS containing 0.05% Tween 20, the antigenic proteins were stained using anti-rabbit IgG antibody conjugated with alkaline phosphatase (diluted 1:2,500). The bands of the proteins were stained with Coomassie Brilliant Blue (Bio-Safe™ Coomassie; Bio-Rad Laboratories, Hercules, CA, USA).
The relative intensity of immunoreactive bands was quantified using ImageMaster 1D Elite software (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and the data were represented as percentage of each control (without ethanol treatment).

**Measurement of protein**

The protein content in the neurons was determined by the previous method (29) using bovine serum albumin as standard after 0.5 M NaOH digested the neurons.

**Statistical analyses**

Each data value was expressed as a mean ± S.E.M. The statistical significance was assessed by the methods described in each figure legend following the application of one-way ANOVA.

**Chemicals**

[^3]H]Diltiazem (3.1 TBq/mmol) and [^45]CaCl₂ (0.3511 GBq/mg) were purchased from PerkinElmer Inc. (Wellesley, MA, USA). (+)-Methyl[^3]H]PN200-110 (3.44 TBq/mmol) was obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). Antibodies against α₁A (catalog No.: ACC-001), α₁B (ACC-002), α₁C (ACC-003), α₁D (ACC-005), and α₂/δ₁ (ACC-015) subunits were products of Alamone Labs, Ltd. (Jerusalem, Israel). Anti-rabbit IgG and antibodies against α₁F (SC-25688) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against α₂ (A6) and β₄ (C-5863) were the products of SWANT (Bellinzone, Switzerland) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. Nifedipine was obtained from Wako Pure Chemical Industries (Osaka). ω-Conotoxin GVIA (ω-CTX) and ω-agatoxin IVA (ω-ATX) were purchased from the Peptide Institute, Inc. (Tokyo). Other reagents used were locally available and of analytical grade.

**Results**

**Changes in 30 mM KCl-stimulated [^45]Ca²⁺ influx after sustained ethanol exposure**

In order to confirm the enhancement of Ca²⁺ influx into the neurons after sustained ethanol exposure, [^45]Ca²⁺ influx was measured. [^45]Ca²⁺ influx stimulated by 30 mM KCl increased with increasing duration of ethanol (50 mM) exposure and the maximal influx was found 3 days after the exposure and thereafter the influx was maintained up to 5 days after the exposure (Fig. 1A). The ethanol exposure showed no changes in the basal influx (Fig. 1A). Therefore, in this study the duration for ethanol exposure was determined to be 3 days. When exposing the neurons to ethanol with various concentra-

![Fig. 1.](image-url)
Effect of inhibitors of HVCCs on 30 mM KCl-stimulated $^{45}\text{Ca}^{2+}$ influx in cerebral cortical neurons in primary culture following exposure to ethanol. The neurons were cultured with 50 mM ethanol at 37°C for 3 days. Each inhibitor for HVCCs was added into the incubation buffer 15 s before the simultaneous addition of 30 mM KCl and $[^{45}\text{Ca}^{2+}]$Cl. The addition of each inhibitor was carried out 15 s before the addition of 30 mM KCl and $[^{45}\text{Ca}^{2+}]$Cl. The neurons without ethanol exposure treatment were used as the control. The $^{45}\text{Ca}^{2+}$ influx without any stimulation by 30 mM KCl was taken as the basal value. Each value represents the mean ± S.E.M. obtained from four separate experiments run in triplicate. **$P<0.01$, Bonferroni’s test. Drug concentrations: $\omega$-ATX, 1 µM; $\omega$-CTX, 1 µM; nifedipine, 1 µM.

Effect of inhibitors of HVCCs on 30 mM KCl-stimulated $^{45}\text{Ca}^{2+}$ influx after sustained ethanol exposure

We investigated which types of HVCCs were concerned in the increase in KCl-evoked $^{45}\text{Ca}^{2+}$ influx. The inhibitors for P/Q- and N-type HVCCs, $\omega$-ATX (1 µM) and $\omega$-CTX (1 µM), respectively, both significantly inhibited the 30 mM KCl-induced $^{45}\text{Ca}^{2+}$ influx into the neurons without ethanol exposure. Similarly, these inhibitors significantly reduced the increases in the KCl-induced $^{45}\text{Ca}^{2+}$ influx in the neurons after ethanol exposure (Fig. 2). However, the levels of the $^{45}\text{Ca}^{2+}$ influx in the ethanol-treated neurons were still higher than the levels in the non-treated neurons (Fig. 2). In contrast, 1 µM nifedipine, an inhibitor for L-type HVCCs, significantly reduced the KCl-stimulated $^{45}\text{Ca}^{2+}$ influx in the ethanol-exposed neurons to a level similar to that of the KCl-evoked $^{45}\text{Ca}^{2+}$ influx in non-treated neurons (Fig. 2), indicating that the sustained ethanol exposure enhances functions of only L-type HVCCs. The basal influx of $^{45}\text{Ca}^{2+}$ was not affected by the treatment with ethanol (Fig. 2).

Changes in $[^{3}\text{H}]$PN200-110 and $[^{3}\text{H}]$diltiazem binding after sustained ethanol exposure

The binding of $[^{3}\text{H}]$PN200-110 to the particulate fractions prepared from ethanol-treated and non-treated neurons consists of a high-affinity binding site. The exposure to ethanol increased $[^{3}\text{H}]$PN200-110 binding, which was due to increased binding sites without any changes in the binding affinity (Fig. 3A).

Figure 3B shows $[^{3}\text{H}]$diltiazem bindings to the particulate fractions from the neurons treated and non-treated with ethanol. $[^{3}\text{H}]$Diltiazem binding in both ethanol-treated and non-treated neurons were saturable and only a high affinity binding site was detected (Fig. 3B). The Scatchard analysis reveals that the sustained ethanol exposure increases the $[^{3}\text{H}]$diltiazem binding with the increase in $B_{max}$ value and no changes in $K_d$ value.

Changes in L-type HVCC subunit expression after sustained ethanol exposure

In order to investigate how the enhancement of L-type HVCC functions is induced, we examined the expressions of subunits of L-type HVCCs as well as P/Q- and N-type HVCCs. As shown in Fig. 4A, the expression of $\alpha_1C$ and $\alpha_1D$ of L-type HVCCs significantly increases after the sustained exposure to ethanol for 3 days, while $\alpha_1A$, $\alpha_1B$, and $\alpha_1F$ subunits for P/Q-, N-, and L-type HVCCs, respectively, showed no alterations (Fig. 4A).

Similarly, the sustained ethanol exposure significantly enhanced the expression of $\alpha_2/\delta_1$ subunit of L-type HVCCs. However, the immunoreactivity against $\alpha_2$ subunit did not show any changes after the exposure to ethanol. The molecular weights of the $\alpha_2/\delta_1$ and $\alpha_2$ subunits were 160 and 135 KDa, respectively (Fig. 4C). The expression of the $\beta_4$ subunit significantly decreased after the sustained exposure (Fig. 4B).

Changes in L-type HVCC subunit expression in the cerebral cortices of alcohol-dependent mice

The control mice administered pyrazole alone for seven days showed no significant alterations in L-type HVCC subunit expressions when compared to those in mice with no treatment (data not shown).

In this study, we also examined how the expression of L-type HVCC subunits with $\alpha_1A$ and $\alpha_1B$ subunits of P/Q- and N-type HVCCs altered in the cerebral cortices of alcohol-dependent mice. As shown in Fig. 5, the expression pattern of various HVCC subunits in the cerebral cortices from mice with ethanol physical dependence was similar to that found in the cerebral cortical neurons continuously exposed to ethanol for 3 days. That is, $\alpha_1C$ and $\alpha_1D$ subunits significantly increased, while $\alpha_1A$ and $\alpha_1B$ subunit expressions showed no alterations in the cerebral cortex from mice with ethanol physical dependence. Moreover, facilitated expression of $\alpha_2/\delta_1$ unit and reduced $\beta_4$ subunit expression with no changes in $\alpha_2$ subunit expression were
noted. These alterations in subunit expressions of P/Q-, N-, and L-type HVCCs are very similar to those in the primary cultures of mouse cerebral cortical neurons after sustained exposure to ethanol as shown in Fig. 4, A and B.

Discussion

In the present study, we have attempted to examine how the expression of L-type HVCC subunits changes to clarify the mechanisms of increased Ca\(^{2+}\) entry into neurons after sustained exposure to ethanol because previous investigations have demonstrated that chronic ethanol treatment increases only the number of binding sites for dihydropyridines, selective inhibitors of L-type HVCCs, and that dihydropyridines partially suppress withdrawal syndrome in human and experimental animals physically dependent on ethanol (3 – 7, 30).

In the present study, we used 50 mM ethanol for sustained exposure to examine changes of L-type HVCCs because the previous studies demonstrated that 50 – 100 mM ethanol in vivo is relevant to the blood ethanol concentrations in clinical populations (31). Moreover, the blood ethanol concentration in animals with ethanol physical dependence induced by sustained ethanol vapor administration employed here was about 290 mg/dl of blood (23), and this blood concentration is considered to be similar to 50 mM ethanol concentration in vitro.

For the purpose described above, the entry of Ca\(^{2+}\) into mouse cerebral cortical neurons in primary culture was examined, at first, by measuring \(^{45}\)Ca\(^{2+}\) influx into the neurons. Our previous reports demonstrated the presence of, at least, four types of HVCCs, P/Q-, N-, and L-type HVCCs, showing responses to depolarization induced by high K\(^+\) (30 mM KCl) (17, 18). This study using inhibitors for various types of HVCCs reveals that the increase in \(^{45}\)Ca\(^{2+}\) influx after sustained exposure to ethanol is due to increased \(^{45}\)Ca\(^{2+}\) entry through L-type HVCCs, and the other types of HVCCs do not participate in the increased \(^{45}\)Ca\(^{2+}\) influx. The enhancement of L-type HVCC functions after chronic ethanol treatment in the neurons used here is in good agreement with the data of the previous reports (3, 5, 6, 32).

The present study demonstrates the increase of the bindings of [\(^3\)H]PN200-110, a dihydropyridine derivative, and of [\(^3\)H]diltiazem after sustained exposure to ethanol. The increased bindings of these radiolabeled ligands were due to the increase of its binding sites, but not changes of their K\(_d\) values. Similar changes with increased binding sites and no alterations in binding affinity of radiolabeled dihydropyridines after long-term exposure to ethanol were reported in animal brain (33, 34) and cultured neural cells (3, 4, 35). In addition, these alterations in the binding sites of labeled L-type HVCC antagonists induced by long-term exposure to ethanol are considered to explain well the increased influx of \(^{45}\)Ca\(^{2+}\). Therefore, the increase in the binding sites means...
Fig. 4. Effect of ethanol exposure on expression of HVCC subunits in cerebral cortical neurons. A: Changes in expression of α subunits. B: Changes in expression of α2, α2/δ1, and β4 subunits. C: Typical presentation of immunoblots of α2 and α2/δ1 units with molecular weight markers. The neurons were cultured with 50 mM ethanol at 37°C for 3 days. Neurons without ethanol exposure treatment were used as the control. Each value represents the mean ± S.E.M. obtained from four separate experiments run in triplicate. C, control (non-treated neurons); E, ethanol-treated neurons. **P<0.01, compared with each control value (without treatment with ethanol, Bonferroni’s test).

Fig. 5. Changes of expression of HVCC subunits in the cerebral cortex from alcohol-dependent mouse. A: Changes in expression of α subunits. B: Changes in expression of α2, α2/δ1, and β4 subunits. Male ddY strain mice weighing 30 g inhaled ethanol vapor containing 120 mg ethanol/10 l of air per min for 8 days with intraperitoneal injection of pyrazole (1.0 mmol/kg per day). Each value represents the mean ± S.E.M. obtained from five mice. **P<0.01, compared with the control (Bonferroni’s test).
the increase in the number of channels through which Ca\(^{2+}\) enters into the neurons.

In this study, we also use \[^{[H]}\]diltiazem, a derivative of benzothiazepines, to examine the changes of L-type HVCC functions because this ligand has the property to bind \(\alpha_1\) subunits of L-type HVCCs as dihydropyridines and phenylalkylamines do, although the binding sites in \(\alpha_1\) subunits of these blockers are different (12, 36). On the other hand, several investigators reported that diltiazem showed somewhat different effects on central actions of ethanol, when compared with those of dihydropyridines (14), and that there was functional interaction between diltiazem and ethanol (35). The latter property of diltiazem was different from that of dihydropyridines (37). The present study demonstrates that chronic ethanol treatment increases the bindings of both \[^{[H]}\]PN200-110 and \[^{[H]}\]diltiazem, and the Scatchard analysis reveals similar mechanisms of increases of bindings, although the rate of increased binding of \[^{[H]}\]diltiazem tends to be smaller than that of \[^{[H]}\]PN200-110.

The immunoblot analysis demonstrates that the expression of \(\alpha_1A\) and \(\alpha_1B\) subunits consisting of P/Q- and N-types of HVCCs, respectively, does not alter after sustained ethanol exposure, which is in good agreement with the data showing that \(^{45}\text{Ca}^{2+}\) entry through these HVCCs after the ethanol treatment shows no alterations. In contrast, results opposite to our data were reported. That is, N-type HVCCs as well as L-type of HVCCs are up-regulated by chronic ethanol treatment of PC12 cells (4, 38) and of striatal synaptosomes from rats (39). This modification of the channels is reported to be due to changing channel kinetics such as fastening activation of the channels (40). Such difference in behaviors of L- and N-type HVCCs to chronic ethanol treatment between these reports and our results may be due to the differences in both cell types used in each experiment and ethanol concentrations exposed, although the exact reasons for such difference are not clear at present.

The physiological roles of \(\beta\) subunits are supposed to increase Ca\(^{2+}\) currents through the ionophores formed by \(\alpha_1\) subunits by modulation of inactivation kinetics and interaction with second messenger regulation (28, 41 – 43). Therefore, it is reasonable to suppose that the decrease in \(\beta_4\) subunit expression may suppress \(\alpha_1\) subunit functions. However, the present study shows the increase in L-type HVCC function, which suggests that the increased expressions of \(\alpha_1C\), \(\alpha_1D\), and \(\alpha_2/\delta_1\) subunits rather than the decrease in \(\beta_4\) subunit expression may affect dominantly the function of L-type HVCCs under the conditions of sustained ethanol exposure. In addition, this study demonstrates that the \(\alpha_1F\) subunit is present in the cerebral cortical neurons and in the cerebral cortex and that chronic ethanol treatment showed no affects on \(\alpha_1F\) subunit expression, although this subunit is reported to be abundantly distributed in the retina (44). The pathophysiological roles of the responses of \(\beta_4\) and \(\alpha_1F\) subunit levels after chronic exposure to ethanol, however, remain to be elucidated.

In both the cerebral cortical neurons continuously exposed to ethanol and the cerebral cortex of mouse with ethanol physical dependence, \(\alpha_2/\delta_1\) subunit expression was enhanced, whereas the level of \(\alpha_2\) subunit showed no changes. The \(\alpha_2/\delta_1\) subunit is post-translocationally cleaved and linked via a sulfide bond (45). This subunit increases the current amplitude (46, 47) and enhances open probability of the channels (48). Therefore, the increased expression of the \(\alpha_2/\delta_1\) subunit may also enhance the function of \(\alpha_1C\) and \(\alpha_1D\) subunits of L-type HVCCs under the alcohol-dependent conditions. On the other hand, the \(\alpha_2\) subunit did not change in spite of the increased immunoreactivity of the \(\alpha_2/\delta_1\) subunit as shown in this study. Both antibodies detecting \(\alpha_2/\delta_1\) and \(\alpha_2\) subunits recognize the different portions on the molecules of \(\alpha_2\) subunits as the product catalogs explain. The molecular weights of \(\alpha_2/\delta_1\) and \(\alpha_2\) subunits were 160 and 135 kDa, respectively, in this study, which is in good agreement with the previous data (\(\alpha_2/\delta\), 160 – 175 kDa; \(\alpha_2\), 135 – 150 kDa) (45, 49). The procedures to determine these subunits in this study have therefore no problems and the detected \(\alpha_2\) subunit may be a reduced \(\alpha_2\) subunit without association with \(\delta\) subunits. In addition, it is reported that the detected \(\alpha_2\) subunits with 135-kDa molecular weight are not associated with \(\delta\) subunits, because the previous investigations demonstrated that the cleavage of \(\alpha_2/\delta\) subunits to \(\alpha_2\) and \(\delta\) subunits occurred (45, 49). Therefore, the difference in the expression of \(\alpha_2/\delta_1\) and \(\alpha_2\) subunits demonstrated here appears to be due to increased expression of \(\alpha_2/\delta_1\) subunit expression with no changes in the rate of proteolytic cleavage of \(\alpha_2/\delta_1\) under the sustained exposure to ethanol, although the exact mechanisms and pathophysiological significance of these events remains unclear at present.

An investigation has revealed that astrocytes have L-type HVCCs and that chronic ethanol treatment induces L-type HVCC up-regulation in these non-neuronal cells (12). More than 95% of the cells used in the present study are proven to be neurons by an immunohistochemical technique, and the other non-neuronal cells are considered to be astrocytes and endothelial cells (15, 16). Therefore, it is reasonable to conclude that the alterations in L-type HVCCs after long-term exposure to ethanol originate from those occurring in the neurons.

The present study using the primary cultures of cerebral cortical neurons demonstrates the increase in
α1C, α1D, and α2/δ1 subunits with decreased α2 subunit, and the alterations in L-type HVCC subunit expression are considered to correspond well to those in the cerebral cortex of mice physically dependent on ethanol, the latter is presented in this study and our previous report (50). These results therefore indicate that the functional modifications of L-type HVCCs after sustained exposure to ethanol in the primary cultures of cerebral cortical neurons reflect the neurochemical changes occurring in L-type HVCCs in the brains from mice with ethanol physical dependence. On the other hand, a previous study reports that chronic exposure to ethanol (120 – 150 mM) produces the increases of α2 as well as α1C and β1 subunits in PC12 cells (51). Such differences in the α2 subunit of L-type HVCCs between PC12 cells and cerebral cortical neurons may be due to the differences in the types of cells used and in the ethanol concentrations for exposure of cells, although the exact reasons are not clear.

In summary, we investigated mechanisms of functional enhancement of L-type HVCCs induced by sustained ethanol (50 mM) exposure for 3 days using mouse cerebral cortical neurons. The treatment with ethanol increased 30 mM KCl-stimulated $^{45}$Ca$^{2+}$ influx, which was abolished by nifedipine, $[^3H]$diltiazem binding with increased $B_{\text{max}}$ value. Western blot analysis showed the increased expression of α1C, α1D, and α2/δ1 subunits with decreased expression of β4 subunit, although expressions of α1A, α1B, and α1F subunits did not alter. These results indicate that sustained ethanol exposure to the neurons induces up-regulation of L-type HVCCs, which was due to increased expression of α1C, α1D, and α2/δ1 subunits and also produces no alterations in the functions of P/Q- and N-type HVCCs. In the cerebral cortex prepared from mice with ethanol physical dependence, a similar pattern of alterations in the expression of various subunits examined was also found. Taken together with these data, the increased expression of the α1C, α1D, and α2/δ1 subunits of L-type HVCCs is an important biochemical event in the cerebral cortex of the brain participating in the development of ethanol physical dependence.

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