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Modification of Nifedipine Inhibitory Effect on Calcium Spike and L-Type Calcium Current by Ethanol in F1 Neuron of *Helix aspersa*

Tourandokht Baluchnejad Mojarad[*1], Mehrdad Roghani[2] and Mahyar Janahmadi[3]

[1]*Dept. of Physiology, School of Medicine, Iran University of Medical Sciences;* [2]*Dept. of Physiology, School of Medicine, Shahed University;* [3]*Dept. of Physiology, School of Medicine, Shaheed Beheshti University of Medical Sciences, Tehran, Iran*

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ABSTRACT

There is strong evidence demonstrating that nifedipine dissolved in ethanol selectively inhibits only L-type Ca^{2+} current. In addition, acute ethanol exposure reduces voltage-dependent calcium currents. In the present study, we investigated the antagonistic effect of fixed concentration of nifedipine dissolved in different concentration of ethanol on L-type Ca^{2+} current. In a Na^+-K^+ free solution, nifedipine dissolved in 60 and 120 mM ethanol decreased resting membrane potential of Ca^{2+} spikes and caused a significant reduction in amplitude, duration and an increase in threshold of Ca^{2+} spikes. Furthermore, Ca^{2+} current was inhibited by ethanol in a concentration-dependent manner, so that the reduction of L-type Ca^{2+} current by nifedipine/60 and 120 mM ethanol was statistically significant. Meanwhile, ethanol concentration-dependent response of Ca^{2+} currents was observed at its late component in more positive potentials. These results may be consistent with ethanol-dependent inhibition of L-type Ca^{2+} currents and ethanol-dependent enhancment of a Ca^{2+}-activated potassium current. *Iran. Biomed. J. 7 (3): 99-105, 2003*

Keywords: Ethanol, Nifedipine, Ca^{2+} spikes, L-type Ca^{2+} current, F1 neuron

INTRODUCTION

Nifedipine, a classical dihydropyridine calcium antagonist has been used to purify and characterize selectively the long-lasting (L-type) calcium channels [1, 2]. As a poorly water-soluble compound, nifedipine is usually dissolved in organic solvents such as acetone [3], dimethylsulfoxide (DMSO) and ethanol [4]. The inhibitory effect of nifedipine is mainly depends on its solvent. In neuroblastoma cells, nifedipine dissolved in ethanol blocked L-type calcium channels but had no effect on T channels [5]. On the other hand, there are many studies demonstrating that several ionic channels are sensitive to pharmacological concentrations of ethanol. The acute ethanol exposure reduces voltage-dependent calcium currents in a variety of systems including dorsal root ganglion cells in rat [6], nerve cells of aplysia [7], rat neurohypophysial terminals [8], PC12 cells [9] and rat pinealocytes [10]. It also inhibits calcium uptake into mouse synaptosomes [11]. The expression of calcium channels in hepatic stellate cells of rat is upregulated by chronic treatment of ethanol [12] and ethanol reduces the duration of single evoked spikes through specific inhibition of voltage-activated calcium currents in acutely dissociated supraoptic neurons in rat [13]. Furthermore, ethanol enhances a calcium-activated potassium current in F1 neuron of *Helix aspersa* [14], isolated neurohypophysial terminals [15], planar lipid bilayer [16] and clonal pituitary (GH3) cells [17]. Since both nifedipine and ethanol individually block calcium currents, it is possible that nifedipine dissolved in ethanol exerts an additive inhibitory effect on L-type calcium channels. The present study is designed to examine the effect of different concentrations of ethanol on antagonistic effect of nifedipine on L-type calcium channels in F1 neuron of *Helix aspersa* using voltage- and current-clamp techniques.

[*]Corresponding Author; Tel. (98-21) 652 2250, Fax: (98-21) 805 8719; E-mail: tmojarad@yahoo.com

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MATERIALS AND METHODS

Animals and dissection. Experiments were performed on the somata of isolated F1 neurons of the sub-oesophageal ganglia of *Helix aspersa* (Iranian garden snail). Specimens were collected locally with average in weight of 4 to 7 g. For dissection, the animal was pinned onto a corkboard in an extended position. The ganglionic mass with its main peripheral nerves and aorta was dissected out and then pinned by the nerves and the edges of the connective tissue into a sylgard-grounded recording chamber (Dowcorning, Midland, MI). The overlying layers of connective tissue covering the ganglia were gently torn using two pairs of fine forceps without any pretreatment with proteolytic enzymes. F1 neurons were visually identified by their size and colour within the right parietal ganglion [18]. F1 neurons were equilibrated for two hours in normal snail Ringer at room temperature (20-23°C) before recording.

Solutions and drugs. The normal snail Ringer contained (in mM): NaCl, 80; $CaCl_2$, 10; KCl, 4; $MgCl_2$, 5; glucose, 10; 4-(2-hydroxyethyl)-1-piperazine-N-ethanesulfonic acid (HEPES), 5; as described by Taylor [19]. The solution used for detection of calcium current contained (in mM): TEA-Cl, 84; $CaCl_2$, 10; KCl, 4; $MgCl_2$, 5; glucose, 10; HEPES, 5.

During recording of calcium currents, 4-aminopyridine (5 mM) and nifedipine were dissolved in different concentrations of absolute ethanol (10, 30, 60 and 120 mM) and were applied to the isolated F1 neurons by bath superfusion. The final concentration of nifedipine in the bath was 1μM in different concentrations of ethanol. The potential-recording and current-passing electrodes were filled with 3 M KCl (pH 7.4). The pH of the solutions was adjusted to 7.8 with either Trizma hydrochloride or Trizma base. All drugs were purchased from Sigma (Sigma, St. Louis, MO, USA). Osmolarity of the solutions was from 212 to 216 mOsm/L of H_2O and it was routinely checked using a Gonotec Osmometer (Osmo Mat 030, UK).

Recording techniques and equipments. A conventional two-microelectrode voltage and current clamp method was applied using Axoclamp-2B amplifier (Axon Instruments, CA, USA). The reference electrode in all experiments was a silver-silver chloride wire within an agar bridge (4% agar in snail Ringer). Microelectrodes were freshly pulled from borosilicate glass capillaries with external diameter of 1 mm and internal diameter of 0.58 mm (Clark Electromedical Instruments, Pangbourne, UK) using a horizontal puller (Stoelting, USA) and then were coated with parafilm. Voltage steps were elicited for 390 ms from holding potentials of -90 mV and -40 mV to various potentials (-90 to +90 or -40 to +90 mV) in 5 mV increments. Command potentials were generated using IBM-compatible computer with Matlab software program. Signals were digitized on-line and stored on the computer for offline analysis. All current traces obtained from voltage clamp experiments were corrected for linear leak and capacity currents. Spontaneous action potential of F1 neuron was recorded in standard Ringer and Na^+ and K^+ free solution before and after adding nifedipine dissolved in differerent concentrations of ethanol. The action potential duration was measured at half peak amplitude.

Data analysis. Data were filtered at 30 KHz. Current and voltage records were sampled at 20 KHz and were digitized online using a 16-bit A/D converter and stored for further analysis. At the end of each experiment, the tip potential was measured and if it was greater than ±5 mV, the related data were discarded. Furthermore, to minimize the errors due to tip potentials, microelectrodes with a low resistance of 6 ± 0.63 MΩ were used. Leak currents were obtained by blocking all known ionic currents with this assumption that the remaining current is leak current. Leak currents were digitally subtracted from the presented data. All values were given as means ± S.E.M. Statistical significance was indicated by $P<0.05$ which was obtained from Student's paired *t*-test and one-way analysis of variance (ANOVA).

RESULTS

The presented data were collected from 42 F1 neurons of parietal ganglion of *Helix aspersa*.

Calcium spikes of F1 neurons. In standard Ringer, F1 neuron exhibited spontaneous firing activity with a direct repolarizing phase of action potential (Fig. 1A). To characterize calcium spikes and their underlying calcium inward currents in somata of F1 neuron, recording was performed through blocking voltage-activated K^+ outward currents (Tetraethyammonium, TEA and 4-Aminopyridine, 4-AP added and/or K^+ omitted from bath) as well as blocking voltage- activated Na^+

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inward currents (Na^+ omitted from bath). Under these conditions, a type of calcium spike with plateau was spontaneously elicited from a holding potential of -35.3 ± 1.40 mV. These spikes showed large amplitude with a plateau phase compared to Na^+ action potential in standard Ringer (85.5 ±3.6 versus 61.53 ± 2.3 mV; Fig. 1B), but like fast action potential were evoked when threshold was exceeded. Since their threshold was near -32.14 ± 1.28 mV, they could be referred to as high-threshold calcium spike (HTS) [20]. Duration of calcium spikes at half spike amplitude was longer than that of Na^+ spikes (229.66 ± 13.33 vs. 6.07 ± 0.89 ms).

The effect of different concentrations of ethanol on antagonistic action of nifedipine on calcium spikes and after spike hyperpolarization. The slow calcium spikes were not observed unless Na^+ and K^+ currents were blocked. Nifedipine (1 μM), an organic blocker of L-type calcium channels dissolved in30, 60 and 120 mM ethanol caused a significant reduction in amplitude (35-40%), duration (17-21%) and an increase in threshold (29-41%) and threshold latency (more than 100%) of calcium spikes and eliminated plateau potential, but did not fully abolish the HTS (Figs. 1C, D and E; Table 1). In standard Ringer, spontaneous action potentials of F1 neuron were followed by a shallow after spike hyperpolarization that its amplitude and duration was 12.56 ± 1.30 mV and 3.78 ± 0.50 ms, respectively (Fig. 1A). In a Na^+-K^+ free solution, after spike hyperpolarization was disappeared, but after application of nifedipine dissolved in 60 and, in particular 120 mM ethanol, a prominent after spike hyperpolarization was appeared following calcium spike (Figs. 1D and E). Amplitude and duration of this after spike hyperpolarization was 7.75 ± 2.09 mV and 54.12 ± 1.07 ms, respectively.

The effect of different concentrations of ethanol on antagonistic action of nifedipine on calcium currents. We have focused on L-type calcium channels in F1 neuron because of its sensitivity to ethanol. In addition, this channel can be easily isolated from other calcium channels using depolarized holding potential of -40 mV.

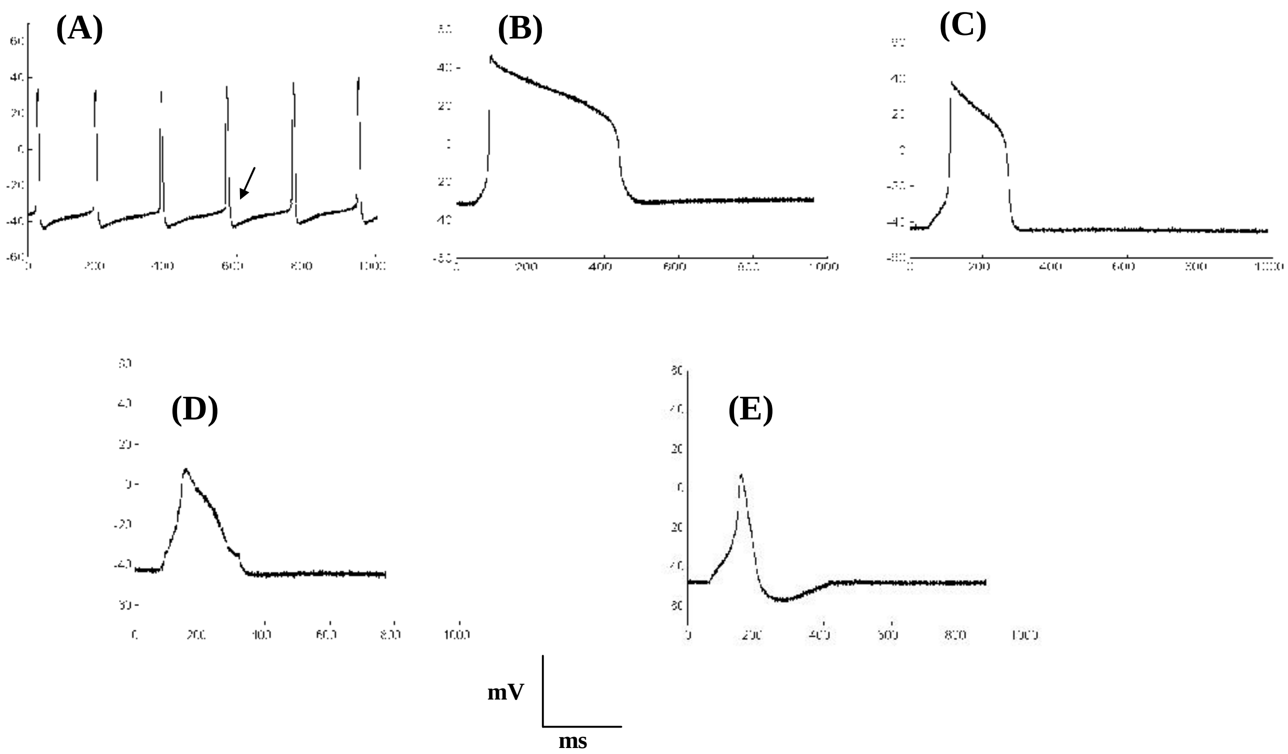

Fig. 1. Intracellular records showing typical spontaneous firing activity in F1 neuron of *Helix aspersa* in standard Ringer **(A)**. In a Na^+-K^+ free solution, spontaneous high-threshold Ca^{2+} spikes were recorded with reduced K^+ currents and blocked Na^+ currents before **(B)** and after adding of nifedipine dissolved in 30 **(C)**, 60 **(D)** and 120 **(E)** mM ethanol. The arrows represent after spike hyperpolarization.

Table 1. The effect of different concentrations of ethanol on Ca^{2+} spike characteristics in F1 neuron. The final concentration of nifedipine in the bath was fixed at 1 μM for each concentration of ethanol.

Membrane electrical properties	Standard Ringer (n = 20)	Na^+-K^+ free Solution (pre- exposure) (n = 14)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (10 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (30 mM) (n = 11)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (60 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (120 mM) (n = 10)
Resting membrane potential (mV)	-37.10 ± 1.1	-31.2 ± 1.60	-36.3 ± 2.30	-42.70 ± 3.70	-42.1 ± 1.1	-47.7 ± 4.90
Action potential amplitude (mV)	61.50 ± 2.3	85.5 ± 3.60	70.3 ± 5.30	67.10 ± 1.10	56.1 ± 2.2*	51.3 ± 2.04*
Action potential duration (ms)	6.07 ± 0.8	229.6 ± 13.30	201.3 ± 10.50	199.01 ± 12.10	191.5 ± 5.4	183.8 ± 4.40*
Action potential threshold (mV)	-33.80 ± 2.1	-32.1 ± 1.28	-30.1 ± 2.18	-29.20 ± 1.29	-22.9 ± 2.3	-19.0 ± 1.60
Threshold latency (ms)	22.70 ± 5.4	53.2 ± 1.20	61.3 ± 2.03	90.50 ± 1.20	92.3 ± 5.2*	107.5 ± 5.89*

**P*<0.05 [compared with characteristics of Ca^{2+} spike in Na^+-K^+ free solution before exposing to nifedipine/ethanol]

Different concentrations of ethanol influences the antagonstic action of nifedipine on L-type calcium channels. After elimination of K^+ and Na^+ currents, we determined the sensitivity of calcium current carried through L-type channels to fixed concentration of nifedipine (1 μM) dissolved in 10-120 mM ethanol using two-electrode voltage clamp. The membrane potential was stepped to +90 mV for 390 ms from holding potentials of -90 and -40 mV in sequential 5 mV increments. The ethanol concentration-dependent response of calcium current is shown in Fig.2. Fig. 2A shows maximum calcium current elicited from a holding potential of -40 mV to +90 mV before and after exposing individual neurons to nifidepine dissolved in different concentrations of ethanol for 5 min and Figures 2B and 2C plot their peak current as a function of voltage (I-V) at holding potentials of -90 and -40 mV, respectively. According to I-V relations, in a Na^+-K^+ free solution, peak calcium current amplitude evoked from -90 and -40 mV was -5.2 ± 0.18 and -4.6 ± 0.33 nA, respectively (Table 2). The I-V relations also showed a characteristic shape indicating several current components at a holding potential of -90 in contrast to -40 mV. The peak current evoked from a holding potential of -90 mV increased gradually with increaseing potential up to about -55 mV, when a plateau was reached. Above -35 mV, the current again increased with increasing potential to reach a maximum at steps up to near -5 mV (Fig. 2B). The I-V relation for peak current evoked from a holding potential of -40 mV showed a single peak near 10 mV (Fig. 2C). Calcium currents were inhibited in a reversible manner by nifedipine dissolved in ethanol (Figs. 2A and B), but this inhibition was not to the same extent in all concentrations of ethanol. Meanwhile, as is shown in the I-V relations, the inhibitory effect of nifedipine dissolved in ethanol has exerted on late component of calcium currents evoked from a holding potential of -90 mV and single peak evoked from a holding potential of -40 mV in more positive potentials (Figs. 2B and C). Peak calcium currents evoked from both holding potentials in neurons exposed to nifedipine/60 mM ethanol and nifedipine/120 mM ethanol were inhibited 60% and 85% respectively (Fig. 2 and Table 2). In all conditions, calcium currents did not reverse from inward to outward below about +30 mV, but the reversal potentials shifted to more negative potentials in an ethanol concentration-dependent manner.

After application of nifedipine/10 mM ethanol, at both holding potentials, the peak current potential shifted to more negative potentials, so that peak current potential evoked from -90 mV before and after application of nifedipine/10 mM ethanol was about 0 and -10 mV and from -40 mV was 15 and

Table 2. The effect of different concentrations of ethanol on peak Ca^{2+} currents at different holding potentials. The concentration of nifedipine in the bath was fixed at 1μM for each concentration of ethanol.

Ca^{2+} current properties	Na^+-K^+ free Solution (pre-exposure) (n = 14)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (10 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (30 mM) (n = 11)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (60 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (120 mM) (n = 10)
Peak Ca^{2+}currents amplitude (nA) -40 mV	-4.6 ± 0.33	-3.7 ± 0.23	-2.9 ± 0.15	-1.9 ± 0.5*	-0.7 ± 0.34*
Peak Ca^{2+}currents amplitude (nA) -90 mV	-5.2 ± 0.18	-3.7 ± 0.23	-2.9 ± 0.15	-2.1 ± 0.2*	-0.98 ± 0.44*

**P*<0.05 [compared with peak Ca^{2+} currents in Na^+-K^+ free solution before exposing to nifedipine/ethanol]

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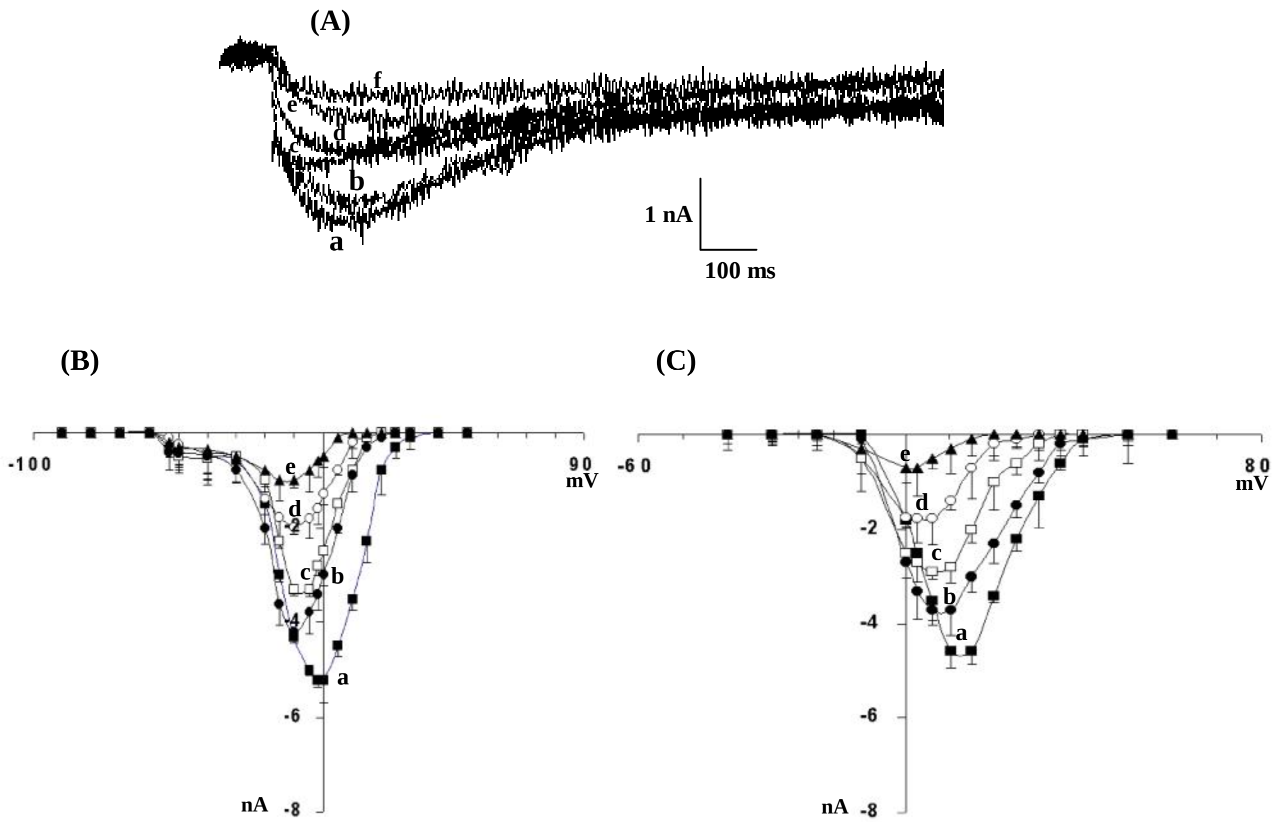

Fig. 2. Ca^{2+} currents in a Na^+-K^+ free solution. **(A)**, Peak current responses in 390 ms voltage steps from a holding potential of -40 mV before **(a)** during **(c-f)** and after (wash; **b**) a 5-min. exposure to 1 μM nifedipine dissolved in 10 **(c)**, 30 **(d)**, 60 **(e)** and 120 **(f)** mM ethanol. **(B)**, I-V relation for the peak currents evoked from a holding potential of -90 mV before **(a)** and after **(b-e)** a 5-min. exposure to 1 μM nifedipine dissolved in 10 **(b)**, 30 **(c)**, 60 **(d)** and 120 **(e)** mM ethanol. **(C)**, I-V relation for the peak currents evoked from a holding potential of -40 mV before **(a)** and after **(b-e)** a 5-min. exposure to 1 μM nifedipine dissolved in 10 **(b)**, 30 **(c)**, 60 **(d)** and 120 **(e)** mM ethanol.

5 mV respectively, but there was no considerable change in peak current voltages with increasing of ethanol concentration.

DISCUSSION

The present study has emphasized on changes in activity of neuronal L-type calcium channels that could be observed at fixed concentration of nifedipine dissolved in different concentrations of ethanol (10-120 mM). In normal Ringer solution, F1 neurons are characterized by spontaneous action potentials associated with short spike duration and after spike hyperpolarization. In a Na^+- K^+ free solution, F1 neurons display high-threshold calcium spikes that are not detected unless Na^+- K^+ currents are reduced. F1 neurons have at least two types of calcium currents: nifedipine-sensitive and nifedipine-insensitive calcium currents. The voltage dependency of the recorded membrane calcium currents indicated two kinds of currents: low-threshold current that is mainly activated near -55 mV and high-threshold current that is mainly activated near -35 mV. That these two current types are carried through different Ca^{2+} channels is supported by the difference in voltage dependency of activation as well as differential sensitivity to nifedipine.

As mentioned previously, nifedipine is a relatively selective blocker of L-type calcium channel but what is unique is the fact that the inhibitory effect of nifedipine is dependent on concentrations of ethanol. The finding of the present study indicated that as increasing concentration of nifedipine dissolved in fixed concentration of ethanol had no effect on low-threshold calcium channels [21], acute exposure of F1 neuron to fixed concentration of nifedipine dissolved in different concentrations of ethanol (10-120 mM) has also no effect on low-threshold calcium channels and specifically blocks only L-type calcium channels but not to the same extent, so that in the presence of ethanol concentrations of 60-120 mM, nifedipine inhibited L-type calcium channels significantly.

Several possible processes could explain ethanol concentration-dependent effect on L-type calcium channels. First, ethanol concentration-dependent inhibitory effect could be due to its direct effect on L-type calcium channels through reduction of effective number of channels or affecting channels gating properties, as many previous studies have also demonstrated that acute ethanol exposure induce reduction of L-type calcium current [9]. On the other hand, the result of previous studies performed on F1 neuron have revealed that in normal physiological solution, ethanol (5-50 mM) hyperpolarized resting membrane potential and depressed both spontaneous action potential duration and its firing rate by enhancement of the repolarization and after hyperpolarization phases respectively [14].

These observations indicate that the major effect of ethanol in concentrations less than 100 mM on activity of F1 neuron may be mediated by potassium currents. Similar findings have been reported elsewhere in tissue slice preparations; in rat hippocampal neurons it was concluded that 5-20 mM ethanol increased a calcium- activated current [22]. It has been suggested that neuronal potassium conductance may be increased by all central nervous system depressant drugs [23], and in particular, intracellular free calcium may be involved in this enhancement [24]. Therefore, it is possible that in addition to direct effect of ethanol on L-type calcium channels, there is an ethanol concentration-dependent enhancing effect on a calcium-activated potassium current. This explanation is strengthened by the fact that after exposure of F1 neuron to nifedipine dissolved in a high concentration of ethanol, a prominent after spike hyperpolarization was appeared and resting membrane potential decreased. Because the reversed pharmacodynamic changes in neuronal ionic channel activity observed when ethanol is chosen as nifedipine solvent, it may be an important application of appropriate concentration of ethanol for an optimal antagonistic effect of nifedipine on L-type calcium channels. Further studies are warranted to investigate the possible mechanisms that are involved in modifying antagonistic effect of nifedipine by its solvent, ethanol.

In conclusion, nifedipine is used extensively in the treatment of cardiovascular and neurological disorders [25]. Nifedipine has been usually dissolved in organic solvents including ethanol. Since ethanol has also direct inhibitory and potentiating effect on membrane ionic currents, for optimal efficacy of nifedipine on L-type calcium channels, it is important that an appropriate concentration of ethanol is used as nifedipine solvent. The present data suggest that increasing of ethanol concentration influences the properties of high-threshold calcium spikes and consequently the neuronal excitability through an ethanol concentration-dependent inhibitory effect on L-type calcium channels and/or ethanol concentration-dependent potentiating-effect on outward potassium current.

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