

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 Roghani² and Mahyar Janahmadi³

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

Received 12 November 2002; revised 7 April 2003; accepted 14 April 2003



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 enhancement 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

Archive of SID

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.

MATERIALS AND METHODS

Animals and dissection. Experiments were performed on the somata of isolated F1 neurons of the suboesophageal 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; CaCl₂, 10; KCl, 4; MgCl₂, 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; CaCl₂, 10; KCl, 4; MgCl₂, 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 1 μM 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 H₂O 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 different 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 4 ± 1 neurons of parietal ganglion of *Helix aspersa*.

Calcium spikes of F1 neurons. In standard Ringer, F1 neuron exhibited spontaneous firing activity with a

Archive of SID

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 (Tetraethylammonium, TEA and 4-Aminopyridine, 4-AP added and/or K^+ omitted from bath) as well as blocking voltage- activated Na^+ 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 spike hyperpolarization. The slow calcium spikes were not observed unless Na^+ and K^+ currents were blocked. Nifedipine, an organic blocker of L-type calcium channels dissolved in 30, 60 and 120 mM ethanol caused a significant reduction (35-40%), duration (17-21%) and an increase in threshold (29-41%) and threshold latency (more than 100%) and eliminated plateau potential, but did not fully abolish the HTS (Figs. 1C, D and E; Table 1). In standard action potentials of F1 neuron were followed by a shallow after spike hyperpolarization that its amplitude and duration were 1.30 mV and 3.78 ± 0.50 ms, respectively (Fig. 1A). In a Na^+ - K^+ free solution, after spike hyperpolarization was not observed 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 were 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.

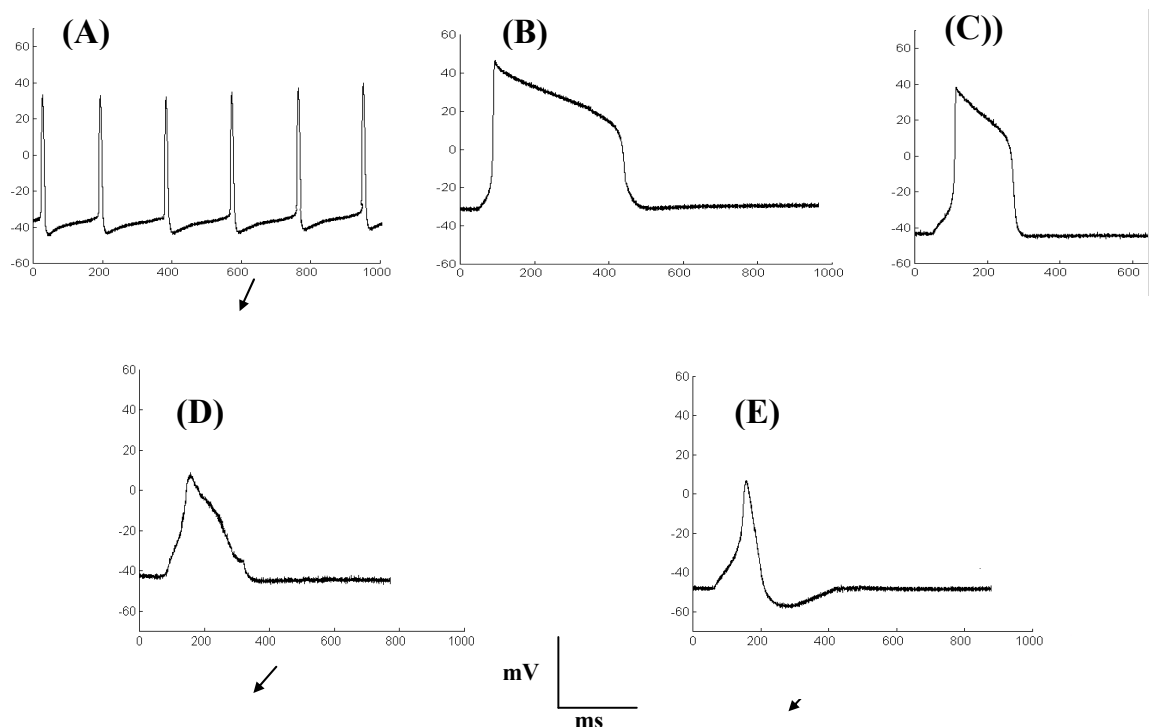


Fig. 1. Intracellular records showing typical spontaneous firing activity in F1 neuron of *Helix aspersa* in standard Ringer (A). Intracellular spontaneous high-threshold Ca^{2+} spikes were recorded with reduced K^+ currents and blocked Na^+ currents before (B) and after adding

Archive of SID

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 properties	electrical	Standard Ringer (n = 20)	$\text{Na}^+ - \text{K}^+$ free Solution (pre-exposure) (n = 14)	$\text{Na}^+ - \text{K}^+$ free Solution + Nifedipine/Ethanol (10 mM) (n = 12)	$\text{Na}^+ - \text{K}^+$ free Solution + Nifedipine/Ethanol (30 mM) (n = 11)	$\text{Na}^+ - \text{K}^+$ free Solution + Nifedipine/Ethanol (60 mM) (n = 12)	$\text{Na}^+ - \text{K}^+$ 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	$56.1 \pm 2.2^*$	$51.3 \pm 2.04^*$
Action potential duration (ms)		6.07 ± 0.8	229.6 ± 13.30	201.3 ± 10.50	199.01 ± 12.10	191.5 ± 5.4	$183.8 \pm 4.40^*$
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	$92.3 \pm 5.2^*$	$107.5 \pm 5.89^*$

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

Different concentrations of ethanol influences the antagonistic action of nifedipine on L-type calcium elimination of K^+ and Na^+ currents, we determined the sensitivity of calcium current carried through L-type 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 potentials of -90 and

-40 mV in sequential 5 mV increments. The ethanol concentration-dependent response of calcium current is shown in Figure 2A shows maximum calcium current elicited from a holding potential of -40 mV to +90 mV before and after exposing individual neurons to nifedipine dissolved in different concentrations of ethanol. Figures 2B and 2C plot their peak current as a function of voltage (I-V) at holding potentials of -90 mV and -40 mV, respectively. According to I-V relations, in a $\text{Na}^+ - \text{K}^+$ free solution, peak calcium current amplitude evoked from a holding potential of -90 mV was -5.2 ± 0.18 and -4.6 ± 0.33 nA, respectively (Table 2). The I-V relations also showed a characteristic shoulder current components at a holding potential of -90 mV. The peak current evoked from a holding potential of -90 mV increased gradually with increasing potential up to about -55 mV, when a plateau was reached. Above this potential, the current again increased with increasing potential to reach a maximum at steps up to near -5 mV (Fig. 2B). The I-V relation 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 shown in Figure 2C, the inhibitory effect of nifedipine dissolved in ethanol has exerted on late component of calcium current evoked from a holding potential of -90 mV and single peak evoked from a holding potential of -40 mV in more positive potentials (Fig. 2C). Peak calcium currents evoked from both holding potentials in neurons exposed to nifedipine/60 mM ethanol were inhibited 60% and 85% respectively (Fig. 2 and Table 2). In all conditions, calcium currents were 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 -15 mV and from -40 mV was -10 mV.

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 1 μM for each concentration of ethanol.

Ca^{2+} current properties	$\text{Na}^+ - \text{K}^+$ free Solution (pre-exposure) (n = 14)	$\text{Na}^+ - \text{K}^+$ free Solution + Nifedipine/Ethanol (10 mM) (n = 12)	$\text{Na}^+ - \text{K}^+$ free Solution + Nifedipine/Ethanol (30 mM) (n = 11)	$\text{Na}^+ - \text{K}^+$ free Solution + Nifedipine/Ethanol (60 mM) (n = 12)	$\text{Na}^+ - \text{K}^+$ 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	$-1.9 \pm 0.5^*$	$-0.7 \pm 0.34^*$
	-5.2 ± 0.18	-3.7 ± 0.23	-2.9 ± 0.15	$-2.1 \pm 0.2^*$	$-0.98 \pm 0.44^*$

Archive of SID

same extent, so that in the presence of ethanol concentrations of 60-120 mM, nifedipine inhibited L-type significantly.

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

These observations indicate that the major effect of ethanol in concentrations less than 100 mM on activity is mediated by potassium currents. Similar findings have been reported elsewhere in tissue slice preparations. In F1 neurons it was concluded that 5-20mM ethanol increased a calcium-activated current [22]. It has been suggested that potassium conductance may be increased by all central nervous system depressant drugs [23], and in particular calcium may be involved in this enhancement [24]. Therefore, it is possible that in addition to direct effect on 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. Based on these results, the pharmacodynamic changes in neuronal ionic channel activity observed when ethanol is chosen as solvent for nifedipine is an important application of appropriate concentration of ethanol for an optimal antagonistic effect of nifedipine on calcium channels. Further studies are warranted to investigate the possible mechanisms that are involved in modifying the effect of nifedipine by its solvent, ethanol.

In conclusion, nifedipine is used extensively in the treatment of cardiovascular and neurological disorders. It has been usually dissolved in organic solvents including ethanol. Since ethanol has also direct inhibitory and potentiating effects on membrane ionic currents, for optimal efficacy of nifedipine on L-type calcium channels, it is important to choose an appropriate concentration of ethanol is used as nifedipine solvent. The present data suggest that increasing of ethanol concentration affects 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 potassium current.

REFERENCES

1. Dale, B., Talevi, R. and DeFelice, L.J. (1991) L-type Ca^{2+} currents in ascidian eggs. *Exp. Cell Res.* 192: 302-306.
2. McKenna, E., Kock, W.J., Sligh, D.F. and Schwartz, A. (1990) Toward an understanding of the dihydropyridine-sensitive calcium channel. *Biochem. Pharmacol.* 39: 1145-1150.
3. Cheng, J.B. and Townley, R.G. (1983) Pharmacological characterization of effects of nifedipine on isolated guinea pig smooth muscle. *Arch. Int. Pharmacodyn.* 263: 228-244.
4. Fox, A.P., Nowicky, M.C. and Tsien, R.W. (1987) Kinetic and pharmacological properties distinguishing three types of voltage-activated calcium channels in chick sensory neurons. *J. Physiol. (London)* 349: 149-172.
5. Wang, R., Karpinski, E., Wu, L.Y. and Pang, P.K.T. (1990) Flunarizine selectively blocks transient calcium channels in rat sensory neurons. *J. Pharmacol. Exp. Ther.* 254:1006-1011.
6. Oakes, S.G. and Pozos, R.S. (1982) Electro-physiological effects of acute ethanol exposure: I. Alteration in the activity of root ganglia neurons in dissociated culture. *Dev. Brain Res.* 281:243-249.
7. Camacho-Nasi, P. and Treistman, S.N. (1987) Ethanol-induced reduction of neuronal calcium currents: an examination of the mechanisms. *Cell Mol. Neurobiol.* 7: 191-207.
8. Wang, X., Lemos, J.R., Dayannithi, G., Nordmann, J.J. and Treistman, S.N. (1991) Ethanol reduces vasopressin-induced calcium currents in nerve terminals. *Brain Res.* 551: 338-341.
9. Mullikin-Kilpatrick, D. and Treistman, S.N. (1995) Inhibition of dihydropyridine-sensitive calcium channels by ethanol in PC12 cells and nerve growth factor-treated PC12 cells: interaction with the inactivated state. *J. Pharmacol. Exp. Ther.* 272: 485-491.
10. Chik, C.L., Liu, Q.Y., Girard, M., Karpinski, E. and Ho, A.K. (1992) Inhibitory action of ethanol on L-type Ca^{2+} dependent guanosine 3', 5'-monophosphate accumulation in rat pinealocytes. *Endocrinol.* 131: 1895-1902.
11. Harris, R.A. and Hood, W.F. (1980) Inhibition of synaptosomal calcium uptake by ethanol. *J. Pharmacol. Exp. Ther.* 214: 100-105.
12. Oide, H., Itatsu, T., Hirose, M., Wang, X.E., Nishiyama, D., Takei, Y. and Sato, N. (2000) Acute and chronic effects of ethanol on L-type calcium channels in hepatic stellate cells. *Alcohol Clin. Exp. Res.* 24: 357-360.
13. Widmer, H., Lemos, J.R. and Treistman, S.N. (1998) Ethanol reduces the duration of single evoked spikes by a voltage-gated calcium currents in acutely dissociated supraoptic neurons of the rat. *J. Neuroendocrinol.* 10: 399-406.
14. Madsen, B.W. and Edson, R.O. (1990) Ethanol enhancement of a calcium-activated potassium current in an identified neuron. *Brain Res.* 528: 323-326.
15. Dopico, A.M., Lemos, J.R. and Treistman, S.N. (1996) Ethanol increases the activity of large conductance, Ca^{2+} -activated potassium current in F1 neurons. *J. Neurophysiol.* 75: 100-105.

Archive of SID

isolated neurohypophysial terminals. *Mol. Pharmacol.* 49 40-48

- 16 Chu, B., Dopico, A.M., Lemos, J.R. and Treistman, S.N. (1998) Ethanol potentiation of calcium-activate reconstituted into planar lipid bilayers. *Mol. Pharmacol.* 54: 397-406.
- 17 Jakab, M., Weiger, T.M. and Hermann, A. (1997) Ethanol activates maxi Ca^{2+} -activated K^+ channels of clonal pi *Membr. Biol.* 157: 237-245.
- 18 Kerkut, G.A., Lambert, J.D.C., Gayton, R.J., Loker, J.E. and Walder, R.J. (1975) Mapping of nerve cells in the suboesophageal ganglia of *Helix aspersa*. *Comp. Biochem. Physiol.* 5A : 1-25.
- 19 Taylor, P.S. (1987) Selectivity and patch measurement of A-current in *Helix aspersa* neurons. *J. Physiol.* 388: 437-447.
- 20 Sundgren-Andersson, A.K. and Johansson, S. (1998) Calcium spikes and calcium currents in neurons from the medial pre-optic nucleus of rat. *Brain Res.* 783: 194-209.
- 21 Wu, L., Karpinski, E., Wang, R. and Pang, P.K.T. (1992) Modification by solvents of the action of nifedipine on ca in neuroblastoma cells. *Naunyn- Schmiedeberg, s. Arch. Pharmacol.* 345: 478-484.
- 22 Carlen, P.L. Gurevich, N. and Durand, D. (1982) Ethanol in low doses augments calcium-mediated mechanisms measured intracellularly in hippocampal neurons. *Science* 215: 306-309.
- 23 Carlen, P.L., Gurevich, N., Davies, M.F., Blaxter, T.J. and Beirne, M.O. (1985) Enhanced neuronal K^+ conductance: a possible common mechanism for sedative-hypnotic drug action. *Can. J. Physiol. Pharmacol.* 63: 831-837.
- 24 Krnjevic, K. (1986) Cellular and synaptic effects of general anesthetics. In: *Molecular and Cellular Mechanisms of Anesthetics*. (Roth, S.H. and Miller, K.W. eds.), Plenum Press, NewYork. pp. 3-16
- 25 Ferrante, J. and Triggle, D.J. (1990) Drug-and disease-induced regulation of voltage-dependent calcium channels. 29 44.