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MODIFICATION OF THE EFFECTS OF DIHYDROPYRIDINES ON VOLTAGE-DEPENDENT CALCIUM CHANNELS IN NEUROBLASTOMA CELLS (N1E-115) BY DIFFERENT SOLVENTS

BY

LINGYUN WU

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Wu Lingyun

(Student's Signature)

Dept. of Physiology
U of A
Edmonton, ALTA. T6G 2H7

(Student's Permanent Address)

Date: June 5, 1991

UNIVERSITY OF ALBERTA

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED **MODIFICATION OF THE EFFECTS OF DIHYDROPYRIDINES ON VOLTAGE-DEPENDENT CALCIUM CHANNELS IN NEUROBLASTOMA CELLS (N1E-115) BY DIFFERENT SOLVENTS** SUBMITTED BY **LINGYUN WU** IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF **MASTER OF SCIENCE**

Dr. P.K.T. Pang (Supervisor)

Dr. E. Karpinski (Co-supervisor)

Dr. R.L. Jones

Dr. K.J. Hutchison

Date: June 5, 1991

DEDICATION

To my husband Rui Wang and my daughter Jennifer Wang for their endurance and support. Without these I cannot achieve my goal.

Also to my parents Wenhua Yi and Xianting Wu with love.

ABSTRACT

Dihydropyridines (DHPs) have been used extensively in the study of voltage-dependent calcium channels (VDCC). Minor changes in the structure of DHPs results in great variations in their effects on VDCC. The activities of DHPs may be changed when dissolved in different solvents. In this study, the whole cell version of the patch clamp technique was used to investigate effects of nifedipine, a DHP antagonist, and Bay K-8644, a DHP agonist, dissolved in different solvents, on the two types of calcium channel currents in neuroblastoma cells (N1E-115). The concentration of all four solvents, dimethylsulfoxide (DMSO), acetone, ethanol (EtOH) and polyethylene glycol (PEG), in the bath was fixed at 0.3% to reach different final concentrations of DHPs. At this fixed solvent concentration, the solvents alone did not have any effect on the transient (T) and the long-lasting (L) calcium channel currents. Both nifedipine and Bay K-8644 dissolved in DMSO decreased T channel currents. This inhibitory effect of DHPs dissolved in DMSO on T channel currents was dose-dependent and reversible. In contrast, T channel currents were inhibited neither by nifedipine dissolved in acetone or ethanol, nor by Bay K-8644 dissolved in EtOH or PEG. Pretreatment of cells with DMSO did not render the T channel current sensitive to Bay K-8644 dissolved in EtOH or PEG, or to nifedipine dissolved in acetone or EtOH. Dried nifedipine/DMSO or Bay K-8644/DMSO powder re-dissolved in acetone, EtOH or PEG inhibited T channel currents. Furthermore, within the concentration range of 10 nM to 100 μM,

nifedipine/DMSO inhibited L channel currents more effectively than nifedipine dissolved in EtOH or acetone. Bay K-8644 (10 nM) increased L channel currents by 80% with DMSO, but only 30% with EtOH, as the solvent. It is concluded that using DMSO as the solvent, nifedipine and Bay K-8644 not only act more effectively on L channels but also block T channels in neuroblastoma cells.

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TABLE OF CONTENTS

List of Abbreviations

cAMP/cyclic AMP	adenosine 3′:5′-cyclic monophosphate
CNS	central nervous system
DHP	dihydropyridines (Bay K-8644 -- calcium channel agonist, nifedipine -- calcium channel antagonist)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DRG	dorsal root ganglia
EtOH	ethanol
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid
FCS	fetal calf serum
G proteins	guanine-nucleotide-binding proteins
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
L channel	long-lasting calcium channel
N channel	neuronal calcium channel
PEG	polyethylene glycol
T channel	transient calcium channel
TEA	tetraethylammonium
Tris	tris(hydroxymethyl)aminomethane

TTX	tetrodotoxin
VDCC	voltage- and time-dependent calcium channels

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Introduction and Literature Review

The change in free intracellular Ca^{++} concentration provides a signal that allows excitable cells, such as nerve and muscle, to respond to external stimuli. One of the major mechanisms for elevating the level of intracellular Ca^{++} is the influx of external Ca^{++} through voltage-dependent calcium channels (VDCC). Consequently, it is very important to understand the pharmacological modulation and functional heterogeneity of voltage-dependent calcium channels.

1.1. Voltage-dependent calcium channels in neuronal cells.

Multiple subtypes of voltage-dependent calcium channels have been identified in neurons (Bossu *et al.*, 1985; Nowycky *et al.*, 1985; Penner and Dreyer, 1986; Fox *et al.*, 1987; Tsien *et al.*, 1988; Dolphin, 1991). In neurons of the dorsal root ganglion (DRG) (Nowycky *et al.*, 1985), three types of calcium channels have been described. They have been named the L, T, and N calcium channels.

Long-lasting (L) channels are activated by strong depolarization (positive to -10 mV) (Nowycky *et al.*,1985; Tsien *et al.*,1988), deactivate rapidly following sudden

repolarization, and inactivate very slowly. The steady-state inactivation of L channels is complete at relatively positive membrane potentials, in the range of 10 to 30 mV. This type of calcium channel is highly sensitive to Cd^{++}, and more permeable to Ba^{++} than Ca^{++}. The unitary conductance for L channels is approximately 25 pS with Ba^{++} as the charge carrier (Nowycky *et al.*, 1985; Cottrell and Green, 1987; Fox *et al.*, 1987). This large unitary conductance is another reason why this type of VDCC is referred to as an " L " channel. In addition, this type of calcium channel in neuronal cells can be blocked by *ώ*-conotoxin (a peptide from the marine snail *Conus geographus*). Other terms such as "slow" or "high-voltage-activated" (HVA) are also used to describe the L channel. Type II channels in N1E-115 cells (Narahashi *et al.*, 1987) are similar to L channels. The L channel seems to be the main pathway for the entry of extracellular calcium into the cytoplasm, fulfilling an essential role in stimulus-secretion coupling and other membrane and metabolic events because of its wide distribution and slow inactivation. The L or type II channel has binding sites for dihydropyridine (DHP) agonists such as Bay K-8644, and DHP antagonists such as nifedipine.

Transient or tiny conductance (T) calcium channels are activated by weak depolarizations (positive to -70 mV) (Fox *et al.*, 1987; Tsien *et al.*, 1988), inactivate quickly, and deactivate moderately following sudden repolarization. Compared to L channels, the steady-state inactivation of T channels is complete at relatively negative membrane potentials, such as from -20 to -30 mV, in N1E-115 cells (Wang *et al.*, 1990). T channels have a similar permeability to Ba^{++} and Ca^{++}, but are

more sensitive to Ni^{++} than to Cd^{++}. The unitary barium conductance of T channels is approximately 8 pS with Ba^{++} as the charge carrier (Fox *et al.*, 1987; Tisen *et al.*, 1988). Furthermore, this type of calcium channel was thought to be resistant to dihydropyridines and ω-conotoxin. The T type of calcium channel is also referred to as the "fast" or "low-voltage-activated" (LVA) channel. In neuroblastoma N1E-115 cells, type I channels have characteristics similar to those of T channels in other tissues (Narahashi *et al.*, 1987). The type I channel inactivates rapidly and completely. The voltage range of activation for the type I channel encompasses the normal resting potential (between -35 and -55 mV) of neuroblastoma cells, so that this channel probably functions at the resting membrane potential of the cell.

The properties of N channels (neither T nor L) are intermediate between T and L channels (Nowycky *et al.*, 1985; Fox *et al.*, 1987; Tsien *et al.*, 1988). Because N channels are observed most often in neuronal cells, the abbreviation of " N " is also used to indicate the ubiquitous "neuronal" distribution of this channel. The N channel differs from the L channel since the macroscopic current inactivates rapidly and is resistant to dihydropyridines (Fox *et al.*, 1987; Tsien *et al.*, 1988; Dolphin, 1991; Swandulla *et al.*, 1991). The N channel differs from the T channel in that it is activated by strong depolarizations (positive to -20 mV) (Fox *et al.*, 1987; Tsien *et al.*, 1988) and is blocked by a low concentration of Cd^{++} and ω-conotoxin. With Ba^{++} as the charge carrier, the unitary conductance of the N channel is approximately 13 pS, which is also between the conductances of L and T channels (Fox *et al.*, 1987; Tsien *et al.*, 1988).

The location of specific types of calcium channels in a particular region of a neuron has been proposed as a mechanism for functional specialization (Miller, 1987; Tsien *et al.*, 1988). However, there is no distinct pattern for the distribution of calcium channel types in neurons.

L channels are found in most of neurons. Their voltage dependence and kinetics are well-suited for transforming the time integral of membrane depolarization into an intracellular Ca^{++} signal to trigger the cellular response. One example of such voltage-response transduction is the release of substance P from DRG neurons (Rane *et al.*, 1987). The electrical role of L channels in neurons is probably minor since it is "quiescent" until the membrane has been highly depolarized.

T channels are often found along with L channels in a wide variety of neurons. In sympathetic neurons, however, such as rat superior cervical ganglion neurons and bullfrog sympathetic neurons only N and L channels have been identified. T channel currents are reported to be absent in these cells (Miller *et al*, 1988). The activation of T channels at very negative membrane potentials makes this type of calcium channel a logical candidate for generation of spontaneous depolarizations and rebound excitation following strong hyperpolarization. T channel activation may be important in determining basal levels of hormone secretion (Cohen and McCarthy, 1987) and for initiating near-threshold membrane phenomena, e.g. speeding up depolarization to threshold after neuronal hyperpolarization (Yaari *et al.*, 1986). In addition, T channels may have some developmental roles since they appear to be the

dominant channel type in embryonic and immature cells such as in undifferentiated N1E-115 cells (Pang *et al.*, 1990). In some neurons, T channels contribute more than L channels to the total Ca^{++} currents moving into the cell (Carbone and Lux, 1986).

N channels exist in motor nerve terminals (Atchison and O'Leary, 1987), synaptosomes (Turner and Goldin, 1985; Reynolds *et al.*, 1986), somas and growth cones of sympathetic neurons (Lipscombe *et al.*, 1988; Thayer *et al.*, 1988). N channels participate mainly in the control of neurosecretion (Tsien *et al.*, 1988). Several reports suggest that N channels cause transmitter release from frog motor nerve terminals and rat sympathetic neurons, since in both systems neurotransmitter release was inhibited by ω-conotoxin but not by nifedipine (Kerr and Yoshikami, 1984; Perney *et al.*, 1986).

The identification of neuronal VDCC as T, L, and N types has helped in understanding the function and modulation of neuronal VDCC. It is, however, worth noting that this classification may be an oversimplification. Although there are many reports demonstrating the existence of T and L channel currents in vascular smooth muscle cells, the clear separation of T and L channels has been challenged. At a holding potential of -80 mV, the transient inward calcium channel currents generated by depolarizing test pulses are not solely composed of T channel currents, and the long-lasting component may also include, to a small degree, some T channel activity (Bolton *et al.*, 1988). The separation of N and L channels in neurons is also not always easy. In addition, without the use of a DHP agonist, such as Bay K-8644, the unitary conductances of N and L channels are nearly similar in some reports (20 and

25 pS, respectively) (Swandulla *et al.*, 1991). Hence, some investigators prefer to use the term "high-voltage-activated calcium channel currents" instead of N and L channels (Regan *et al.*, 1991). Even the high-voltage-activated calcium channels show a tissue selective sensitivity to calcium channel antagonists or toxins. For instance, although ω-conotoxin inhibits peripheral neuron calcium channel currents (N or L) to a great extent, the same calcium channel currents in the central nervous system (CNS) neurons are often less affected by this toxin (Dolphin, 1991). Llinas *et al.* (1989a) reported another type of VDCC in Purkinje cells, i.e. P channels. These channels are activated by strong depolarization, and are insensitive to both DHPs and ω-conotoxin. The polyamine toxin from the funnel web spider is an antagonist of these channels (Llinas *et al.*, 1989b).

In the present study, murine neuroblastoma cells (clone N1E-115) are used. This cell preparation is a transformed mammalian sympathetic line, derived from C1300 mouse tumor cells and adapted for growth *in vitro* (Amono *et al.*, 1972). N1E-115 cells possess considerable advantages over other vertebrate neurons. For example, they are easy to differentiate using dimethylsulfoxide (DMSO) (Kimhi *et al.*, 1976). Two types of voltage-dependent calcium channels, T and L, have been identified in this cell preparation using the patch clamp technique (Narahashi *et al.*, 1987). Using the Arsenazo III absorbance technique, two components of voltage-dependent calcium influx were also demonstrated in this cell line (Bolsover, 1986). In contrast, only the L channel component was detected in normal frog sympathetic neurons (Smith *et al.*, 1983). The separation of T and L channels in N1E-115 cells

is relatively easy. Pang *et al.* (1990) reported that if these undifferentiated cells were grown in normal medium, only T channel currents could be detected. When 2% DMSO was included in the media for an extended period of time, L channel currents were most often recorded. The N1E-115 cell is an adrenergic clone since it contains tyrosine hydroxylase activity (Amano *et al.*, 1972). Therefore, this cell can be used as a model to examine the modulation of neurotransmitter release (Tsunoo *et al.*, 1986). The N1E-115 cell, *in vitro*, is an ideal model for analysis of electrical properties of the neuronal membrane because of its large size and its ability to maintain a stable current for hours (Nelson, 1977). Using this model the effects of drugs can be studied and biochemical and electrophysiological measurements can be correlated (Freedom *et al.*, 1984; Quandt and Narahashi, 1984).

1.2. Dihydropyridines and their effects on calcium channels

Calcium antagonists are agents which act on voltage-dependent calcium channels and decrease the inward movement of Ca^{++} through these channels (Janis and Triggle, 1990). Calcium antagonists are further divided into two groups (Godfraind, 1987). Group I is composed of the selective calcium entry blockers. Among four major classes of Ca^{++} channel antagonists in group I are the dihydropyridines (i.e. nifedipine and nimodipine) (Greenberg, 1987). The other classes include the phenylalkylamines (i.e. verapamil), the benzothiazepines (i.e. diltiazem), and the diphenylpiperazines (i.e. flunarizine). Group II calcium antagonists are nonselective calcium entry blockers. Group IIA includes agents

which act at similar concentrations to inhibit both calcium channels and fast sodium channels. Benecyclane is one of the antagonists which is included in Group IIA. Group IIB is composed of agents which interact with calcium channels and have another primary site of action (Godfraind, 1987). Among the targets of Group IIB agents are catecholamine receptors and, opiate receptors. Benextramine and papaverine are examples of Group IIB agents. In the following sections, the literature review will focus on DHPs and their effects on VDCC.

Dihydropyridines are a group of drugs which have similar molecular structures based on phenyl and dihydropyridine rings. There are only small molecular differences between agonists and antagonists derived from the same 1,4-DHP molecule. Agonist activity is generally associated with the S-enantiomers and antagonist activity with the R-enantiomers (Hof *et al.*, 1985; Bechem *et al.*, 1988; Triggle *et al.*, 1989). The presence of the C-5 NO_2 or lactone groups generates the most potent activator series (Kwon *et al.*, 1989). DHP stereoselective effects on calcium channels have been demonstrated with the S-enantiomers being much more potent than R-enantiomer at the activator site. S- and R-enantiomers are approximately equipotent at the antagonist site. The C-5 substituent of 1,4-DHPs is important for the determination of the activator properties through a vectorial interaction between the DHP molecule and, possiblely, the voltage-sensor component of the channel (Holtje and Marrer, 1987). However, agonist properties are also observed in non-chiral 1,4-DHPs including the potent antagonists with ester functions at C-3 and C-5.

DHP calcium channel antagonists are employed in the treatment of a number of cardiovascular disorders including angina in its several forms, hypertension, peripheral vascular disorders and some types of cardiac arrhythmias (Olivery *et al.*, 1979; Greenberg, 1987; Ferrante and Triggle, 1990; Janis and Triggle, 1990). Nimodipine is the first DHP calcium channel antagonist approved for the treatment or prevention of neurological defects associated with subarachnoid hemorrhage (Janis and Triggle, 1990). The theoretical basis for the use of DHP antagonists is that this group of agents specifically blocks the L type, but has no effect on the T type calcium channel. Vasodilation and decreased cardiac output are usually the expected result of clinical use of DHP antagonists.

The existence of a specific DHP receptor in cardiac muscle, smooth muscle, and neuronal cells has been demonstrated using ^{3}H-labelled nitrendipine or nifedipine (Bellemann *et al.*, 1981; Holck *et al.*, 1982; Schramm *et al.*, 1983; Borsotto *et al.*, 1985; Kwon *et al.*, 1989). Kwon *et al.* (1989) reported that a series of DHP compounds, such as Bay K-8644, nifedipine and nitrendipine, had similar binding affinities in smooth muscle, cardiac muscle and neuronal preparations. This suggests that DHP agonists and antagonists may interact at or near common binding site(s) which are components of a VDCC (Schramm *et al.*, 1983; Kwon *et al.*, 1989).

The molecular nature of DHP-sensitive calcium channels has been actively studied. Progress achieved in this area is due to the use of multidisciplinary techniques (i.e. techniques from pharmacology, biochemistry, immunology, and molecular biology). However, the crucial link in the purification of L channels is the

use of tritiated 1,4 DHPs due to their high- and specific-affinity for calcium channels/DHP receptors. Five putative subunits of DHP-sensitive calcium channels have been obtained from of skeletal muscle preparations. They are α_1, α_2, β, γ, and δ subunits (Catterall, 1988; Glossmann and Striessnig, 1988; Mckenna *et al.*, 1990) The full-length amino acid sequences of α_1, α_2, and β have been deduced from cDNA clones (Tanabe *et al.*, 1987; Ellis *et al.*, 1988; Ruth *et al.*, 1989). The important functional roles of the α_1 subunit have been demonstrated. Injection of skeletal muscle α_1 subunit cDNA into dysgenic mouse cells, which do not have functional L channels, restored excitation-contraction coupling and dihydropyridine-sensitive calcium channel activity (Tanabe *et al.*, 1988). The roles of the other putative subunits are still unclear. It appears that the α_1 subunit from either heart or skeletal muscle is sufficient to form an L channel and to bind 1,4 DHP (Perez-Reyes *et al.*, 1989). Finally, specific sites of phosphorylation which seem to be involved in L channel regulation have been discovered (Catterall, 1988; O'Callahan *et al.*, 1988). The phosphorylation of α_1 and β subunits of the purified L channel protein is related to the L channel conductance (Nunoki *et al.*, 1989). If subunits are not phosphorylated, the channel can not be activated.

The sensitivity of the VDCC to DHPs has been considered to be the most important pharmacological criterion used to identify different types of calcium channels. L channel currents are very sensitive to dihydropyridines. DHP antagonists, e.g. nifedipine, nimodipine and nitrendipine, inhibit L channel currents (Bean, 1984; Hess *et al.*, 1984; Fox *et al.*, 1987; Wang *et al.*, 1990). Minor differences

in the structure of dihydropyridines results in a great difference in their effects on VDCC. For example, if at position 5 on the pyridine ring, the carbomethoxy is replaced by a nitro group and at position 2 on the phenyl ring, a nitro group is replaced by trifluoromethyl, a new compound, i.e. Bay K-8644, is thus derived from nifedipine. This minor structural modification converts a calcium channel antagonist (nifedipine) into an agonist (Bay K-8644) which enhances rather than inhibits calcium channel currents (Schramm *et al.*,1983; Freedman and Miller, 1984). Other DHP agonists include CGP 28392, YC-170, and 202-791 (Brown *et al* 1984; Hess *et al.*, 1984; Ochi *et al.*, 1984; Fox *et al.*, 1987; Pang *at al.*,1990). ω-conotoxin can block both N and L channels in neurons (Tisen *et al.*, 1988; Dolphin, 1990; Swandulla *et al.*, 1991). However, N channels are peculiar in their insensitivity to dihydropyridines and sensitivity to Cd^{++} (Dascal, 1990). T channels were believed to be resistant to dihydropyridines. It has been, however, reported that nifedipine could block not only L channels but also T channels in hypothalamic neurons (Akaike *et al.*, 1989). Loirand *et al.* (1989) found that isradipine decreased both T and L calcium channel currents in smooth muscle cells. Felodipine was also reported to inhibit T channel currents in GH_3 cells (a pituitary tumor line) (Van Skiver *et al.*, 1989). In addition, neuronal T channel currents have been reported to be blocked by gallopamil (hypothalamic neurons; Akaike *et al.*, 1989), amiloride (neuroblastoma and chick dorsal root ganglion neurons; Tang *et al.*, 1988), chlorpromazine (neuroblastoma cells; Ogata and Narahashi, 1990), and nicergoline (hippocampal CA1 pyramidal neurons; Takahashi and Akaike, 1990). Flunarizine has been demonstrated to be a

T channel-specific blocker in neuroblastoma cells (Wang *et al.*,1990). Akaike *et al.* (1989) showed that flunarizine was not specific but blocked both L and T channels in hypothalamic neurons.

Many different approaches have been used to modify the activities of DHPs. Manian *et al.* (1987) reported that changes in the substitution pattern at the C-3, C-4 and C-5 positions of the nifedipine analogue altered the conformation, activity, and tissue selectivity of this agent. The activities of DHP antagonists have also been demonstrated to be highly dependent on their size (Rodenkirchen *et al.*, 1979). Furthermore, it was found that the most active compounds showed the smallest degree of ring distortion from planarity (Janis and Triggle, 1983; Fossheim, 1986). Since DHPs are insoluble in water, they are often dissolved in organic solvents. Different solvents may also exert some effects on the conformation of DHPs. Although no attention has been paid to the effect of solvents on DHPs, this may be an important factor. This topic is reviewed in detail in the following section.

1.3. The effect of solvents on voltage-dependent calcium channels

Ethanol, DMSO, acetone, and polyethylene glycol (PEG) are the common solvents which are widely used to initially dissolve DHPs in pharmacological experiments.

1.3.1. Ethanol

Ethanol has been reported to inhibit neuronal calcium channels (Messing *et*

al., 1986; Skattebol and Rabin, 1987). A low concentration of ethanol (25 mM) inhibited the "fast-phase" calcium uptake in potassium-depolarized synaptosomes (Leslie *et al.*,1983). Camacho-Nasi and Treistman (1986) reported that 50 mM ethanol caused a 20 percent inhibition of calcium currents in *Aplysia* neurons. In addition to its direct effect on calcium channels, ethanol may inhibit calcium entry through the elevation of intracellular calcium which may, in turn, inactivate calcium channels (Nashi and Oyama, 1983). Shah and Pant (1988) found that ethanol (30-500 mM) produced a concentration-dependent release of calcium from microsomes. This action of ethanol appears to result from a direct effect of ethanol on calcium release rather than a indirect mechanism induced by an ethanol-elicited increase in the level of inositol triphosphate (Machu *et al.*, 1989). In addition, using the fluorescent indicator fura-2, Daniell *et al.* (1987) reported that ethanol increased cytosolic calcium. In the absence of extracellular calcium, a significant elevation of cytosolic calcium was observed in the presence of ethanol using concentrations of 50 mM or more. At concentrations higher than 100 mM, ethanol-induced increases in intracellular free calcium were also observed in PC12 cells (rat phenochromocytoma cell line) (Rabe and Weight, 1988). Recently, Twombly *et al.* (1990) studied the effect of ethanol on type I and type II voltage-dependent calcium channels in N1E-115 cells using the whole-cell voltage clamp technique. They found that ethanol at a concentration of 100 mM blocked the calcium channel currents (type I and II) by approximately 15%. There was no difference in the dose-related inhibition of type I or type II calcium channel currents by ethanol.

1.3.2. DMSO

DMSO has been reported to facilitate the differentiation of neuroblastoma cells (Narahashi *et al.*, 1987) and HL-60 cells (human promyelocytic leukemia cells) (Baud *et al.*,1987). Pang *et al.* (1990) found that after one month of culture in media containing 2% DMSO, T channel currents were no longer detectable in neuroblastoma cells (N1E-115). An inward current which was due to the L channel was the predominant component. Brown *et al.* (1990) used the calcium indicator Arsenazo III to demonstrate that 5% (v/v) DMSO increased intracellular Ca^{++}. Furthermore, Barrett *et al.* (1986) reported that DMSO alone increased the contraction of canine coronary arterial rings in response to anoxia and 5-hydroxytryptamine. This effect of DMSO was ascribed to the increased influx of extracellular calcium. In the same study, nifedipine dissolved in DMSO was reported to inhibit this anoxia and 5-hydroxytryptamine induced contraction. In order to study the ionic mechanisms underlying the modulation of synaptic transmission, Rahamimoff *et al.* (1988) fused purified cholinergic synaptosomes from the electric organ of *Torpedo* into spherical structures (20 to 50 μm in diameter) using fusion-inducing medium which contained 20% DMSO. A large conductance K-preferring channel (P channel), which is different from the P type of calcium channel reported by Llinas *et al.* (1989 a,b), was recorded in the fused synaptosome preparation. These K-preferring P channels were not observed in the fused preparation of non-synaptic membrane. This study suggests that DMSO at high concentrations dissolves the membrane non-specifically but does not affect functional channel proteins.

1.3.3. Polyethylene glycol (PEG) and Acetone

PEG at the concentration of 0.1% (v/v) had no effect on calcium channel currents in DRG (Regan *et al.*, 1991). At high concentrations (25% v/v), PEG had a non-specific membrane dissolving effect (Rahamimoff *et al.*, 1988). There have been no reports describing the effects of acetone on T and L calcium channel currents.

1.4. Influence of solvents on the effects of dihydropyridines

As a group of poorly water soluble compounds, DHPs are usually dissolved in organic solvents such as acetone (Godfraind 1983; Cheng and Townley 1983; Wang *et al.*, 1990), DMSO (Barrett *et al.* 1986), ethanol (Arreola *et al.* 1987; Fox *et al.* 1987), and PEG (Bean *et al.*, 1986). These solvents may modify the properties of the DHPs. With ethanol or acetone as the solvent, nifedipine was reported to inhibit the L channel current but had no effect on the T channel current in chick dorsal root ganglia (Fox *et al.*, 1987), neuroblastoma cells (Wang *et al.*, 1990), and frog skeletal muscle (Almers *et al.*, 1981; Arreola *et al.*, 1987). Nishi *et al.* (1983) used nifedipine·HCl dissolved in bath solution (extracellular) and reported that it blocked the L but not T channel currents in helix neurons. Uehara and Hume (1985) described the inhibition of inward Ca^{++} currents in frog atrial cells by nifedipine dissolved in polyethylene glycol. However, considering the very negative holding potential (-90 mV), the fast inactivation, the I-V relationship and steady-state inactivation of the Ca^{++} current in their experiments, it is likely that this Ca^{++}

current was similar to the classical T channel current. Hering *et al.* (1988) reported that nifedipine blocked both transient and long-lasting components of Ca^{++} channel currents by binding only to the closed but available (not inactivated) state of channels in vascular smooth muscle cells (no description of the solvent was given). Nifedipine-induced inhibition of both L and T channels was also observed in hypothalamic neurons (Akaike *et al.* 1989). The controversial reports regarding the effect of nifedipine and other dihydropyridine antagonists (Loirand *et al.* 1989; Van Skiver *et al.* 1989) on T channel currents are unpredictable and difficult to explain. The working hypothesis of this thesis is that nifedipine and other dihydropyridines can affect T channel currents but this effect depends, at least in part, on the solvent used.

With ethanol as a solvent, Bay K-8644 was reported to increase the amplitude of L-type calcium channel currents in chick sensory neurones (Fox *et al.*, 1987) and neuroblastoma N1E-115 cells (Pang *et al.*, 1990; Wang *et al.*,1990). In cultured cardiac cells exposed to Bay K-8644 dissolved in ethanol, there is an increase in the mean open time of the long-lasting Ca^{++} channel when single-channel currents are recorded (Kokubun and Reuter, 1984). Using Bay K-8644 dissolved in PEG, Bean *et al.* (1986) found that this agent selectively increased L channel currents in vascular smooth muscle cells. However, Bay K-8644 dissolved in PEG had little or no effect on the transient calcium channel (T channel) or neuronal calcium channel (N channel) currents (Nowycky *et al.*, 1985). All of these studies support the notion that Bay K-8644 is a specific L channel agonist. There is no report regarding the use of

acetone to initially dissolve Bay K-8644.

Bay K-8644 is usually supplied as a racemic mixture of two optical isomers (enantiomers) with (-)Bay K-8644 being the L channel agonist and (+)Bay K-8644, the L channel antagonist (Kass *et al.*, 1987). The racemic mixture of Bay K-8644 has been reported to be an L channel agonist (Fox *et al.*, 1987; Wang *et al.*, 1990) without any effect on T channels. Another example of such a DHP racemic mixture is 202-791. One of its enantiomers is a VDCC antagonist, and the other is a VDCC agonist (Miller, 1987). However, it should be noted that not all enantiomers of DHPs have opposing effects on calcium channels. Both the racemic mixture nimodipine, and its enantiomers (+)nimodipine and (-)nimodipine, inhibited calcium channel currents (Valdivia and Coronado, 1990). Franckowiak *et al.* (1985) studied the effect of optical isomers of Bay K-8644 dissolved in DMSO on isolated rabbit aortic and heart preparations. They reported that (-)Bay K-8644 had the known vasoconstricting and positive inotropic properties of Ca^{++} agonistic agent, while its antipode (+)Bay K-8644 (10-50 times higher concentrations) showed the vasodilating and negative inotropic effects of Ca^{++} antagonistic agents. Moreover, using the patch-clamp technique, Kass *et al.* (1987) using cardiac cells reported that (+)Bay K-8644 (Bay K-4427) inhibited L channel currents, while (-)Bay K-8644 (Bay K-5417) enhanced or inhibited L channel currents. (-)Bay K-8644 caused an increase in L channel currents with the holding potential at -80 mV and a decrease when the holding potential was more positive (-30 mV). In these studies conducted by Kass *et al.* (1987), the relationship between the effect of enantiomers of Bay K-8644 and

solvents was not discussed. Using ethanol as the solvent, (-)Bay K-8644 was reported to potentiate KCl-induced contraction of rat tail artery strips while (+)Bay K-8644 inhibited this contraction (Wei *et al.*, 1986). All of the above studies suggest that the inherent properties of both agonists and antagonists of calcium channels may reside within the same DHP molecules even though not all DHPs have this feature.

1.5. Objectives

The hypothesis that forms the basis of the thesis is that the effects of dihydropyridines on voltage-dependent calcium channels in neuroblastoma cells may be modified by solvents. Nifedipine and Bay K-8644 are used since the former is a typical DHP antagonist and the latter is a representative DHP agonist.

1.5.1. Investigation of the effects of nifedipine and Bay K-8644 dissolved in different solvents on T channel currents.

Traditionally, T channels were known as DHP resistant. This DHP insensitivity has been used as the key criterion in identifying T channels. However, several recent studies show the inhibition of T channels by DHPs. This unpredictable DHP sensitivity of T channels may be related to the solvents used. Once the solvent effects on the action of DHPs are determined, the DHP sensitivity of T channels may be established and become predictable.

1.5.2. Confirmation and extension of the classical effects of nifedipine and Bay

K-8644 dissolved in different solvents on L channel currents.

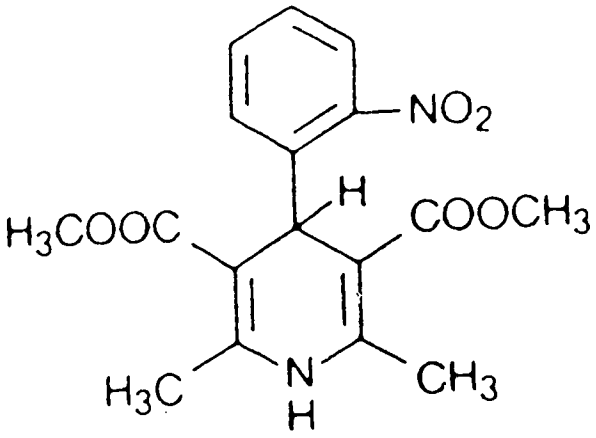
Since the effects of DHPs on L channels are well studied, the emphasis of this thesis study is on the novel effect of DHPs on T channel currents. If solvents modify the action of DHPs on T channels via a direct effect on the structure of DHP molecules, this modified DHP molecule, even if the modification is quite minor, may also exhibit a difference in its effect on L channels. The investigation of the effects of solvents on the action of DHPs on L channels will provide additional evidence to help understand the effect of DHPs on T channels. Ethanol or acetone is the solvent used most often for nifedipine and ethanol for Bay K-8644. In addition, the effects of nifedipine and Bay K-8644 dissolved in ethanol or acetone on L channel currents have been well documented in literature. Hence, the focus of the present investigation is to study the effects of nifedipine and Bay K-8644 dissolved in DMSO, and to compare these results with those in which ethanol or acetone is used as the solvent.

1.5.3. Investigation of the potential interaction among solvents, calcium channels, and DHP molecules.

If nifedipine or Bay K-8644 dissolved in different solvents has different effects on T- and L- types of calcium channels, there are two possibilities to explain these effects. First, solvents alone may have effects on the VDCC. In case the functional state of the calcium channel in the cell membrane is modified by solvents, the calcium channel may become sensitive or more sensitive to nifedipine or Bay K-8644.

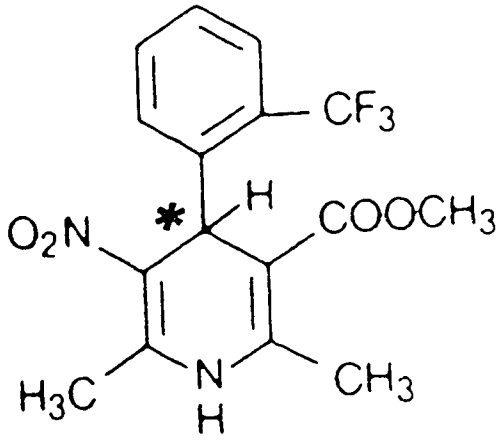
Second, solvents may directly modify the activities of nifedipine and Bay K-8644 on VDCC by changing either the solubility or the structure of the DHPs. These possibilities will be explored in the present study.

A.



Nifedipine

B.



Bay K-8644

Fig.I-1. Chemical structures of two dihydropyridines. **A.** nifedipine (4-(2′-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine. **B.** Bay K-8644 (2,6-dimethyl-3-carbomethoxy-5-nitro-4-(2-trifluoromethyl-phenyl)-1,4 -dihydropyridine. * Denotes the asymmetric centre in the chemical structure of Bay K-8644.

CHAPTER 2

Materials and Methods

2.1. Cell preparation.

Mouse neuroblastoma cells (clone N1E-115), which are a sympathetic neuron tumor line, were obtained from Dr. David Schiff (Department of Paediatrics, University of Alberta) and were cultured using conventional tissue culture techniques. Cells used in the present study were from passages 28-50, which were subcultured on a weekly basis with a splitting ratio of 1:5. The cells were maintained in a humidified atmosphere containing 5% CO_2 in room air at 37°C. The culture medium used was Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, U.S.A.) supplemented with 10% fetal calf serum. This medium was changed every 3 or 4 days and the cells were used before they became confluent. After mechanical agitation, the cells were replated on 35 mm tissue culture dishes. Four to six hours after replating, the attached single cells were used for electrical recording. The cells cultured as described above were spheroidal in shape with a diameter of approximately 25 μm, and had no visible outgrowths or neurites. Predominantly T channel or type I currents (Narahashi *et al.* 1987) were recorded in these N1E-115

cells.

In experiments in which L channels were studied, the cells were grown and maintained at confluence for 3-4 weeks under the same culture conditions as above except that 2% DMSO (v/v) was added to the DMEM. This manipulation was conducted to induce cell differentiation and expression of the characteristics of mature neurons (Kimhi *et al.*, 1976; Moolenaar and Spector, 1978). The cells were replated 3 to 5 days before use in a Petri dish and maintained in DMEM with 2% DMSO. Although many cells had several short processes, only those with processes shorter than the cell diameter were used for patch clamp experiments in order to achieve uniform spatial control of the membrane potential. The mean diameter of these cells was approximately 36 μm. Predominately L channel or type II currents (Narahashi *et al.*, 1987) were recorded in these cells (Pang *et al.* 1990).

The medium in culture dishes with attached cells was replaced by 3 ml external recording solution before every experiment.

2.2. Patch clamp technique.

2.2.1. Solutions.

The external and internal solutions used in this study were designed to isolate voltage-dependent calcium channel currents from other ionic currents. The extracellular solution (bath solution) used for recording the inward calcium channel current was of the following composition (in mM): $BaCl_2$ 20, Tris 105, KCl 5, CsCl 5, HEPES 20, glucose 20 and tetrodotoxin (TTX) 0.5 x 10^{-3}. In all experiments,

barium was used as the charge carrier instead of calcium. The reasons for using barium are listed as follows. 1) This divalent ion is more permeable than calcium through voltage-dependent calcium channels (L and N) (Fox *et al.*, 1987; Tsien *et al.*, 1988; Yoshii *et al.*, 1988). 2) Barium prevents calcium-induced calcium channel inactivation (especially for L channels). It has been reported that the influx of barium during repeated depolarization did not have a significantly inhibitory effect on calcium channels (Brown *et al.*, 1981; Lee *et al.*, 1985; Ohya *et al.*,1988). 3) Barium suppresses K^+ currents bidirectionally. Although barium had been reported to increase the potassium conductance (Meech and Standen, 1975), most of the recent studies have demonstrated that a high concentration of barium in the external solution actually blocked the calcium-activated potassium channel (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Benham *et al.*, 1985). In addition, barium suppressed potassium currents through the delayed rectifier channel (Armstrong *et al.*, 1982) and the inward rectifier channel (Hagiwara *et al.*, 1978). 4) The use of barium in the bath will also block Ca^{++}-activated Cl^- channels (Akbarali *et al.*, 1991).

The Na^+-free bath solution used in these experiments also contained TTX in order to eliminate any possible inward currents through Na^+ channels. Tris replaced Na^+ in the bath solution, and has been shown to reduce leakage currents (Moolenaar and Spector, 1978).

The pipette (internal) solution contained (in mM): CsCl 130, ATP-Na_2 2, HEPES 20, glucose 5, $MgCl_2$ 5, cAMP 0.25, and EGTA 10. Cesium ions blocked K^+ channels (Klöckner and Isenberg, 1985). ATP and cAMP in the internal solution

were used in an attempt to delay the "run down" of the calcium channels (Fedulova *et al.*, 1985). Since 10 mM EGTA was included in the Ca^{++}-free pipette solution, the concentration of intracellular free calcium was estimated to be less than 10^{-9} M (Hagiwara and Byerly, 1981; Stockbridge, 1987; Sada *et al.*, 1988). The low concentration of intracellular calcium will help to decrease the calcium-induced inactivation of calcium channel currents.

In order to ensure the inhibition of outward K^+ channel currents, 20 mM tetraethylammonium chloride (TEA) was also added to the bath solution in some experiments. TEA blocks delayed rectifier and Ca^{++}-activated K^+ channels (Fishman and Spector, 1981; Hugues *et al.*, 1982). There was no measurable difference in the inward Ba^{++} currents recorded with or without TEA in the solution, indicating that Cs^+ was effective in inhibiting K^+ currents. At the end of most experiments, 2 mM La^{+++} was added to the bath solution and the recorded inward currents were completely eliminated. This indicated that the inward currents were due to the inward movement of Ba^{++} via calcium channels. In addition, in some experiments a high concentration of Ba^{++} (50 mM) or different concentrations of Ca^{++} (2.5 or 20 mM) were used in Na^+-free bath solutions. Under these conditions, the inward current amplitude was changed correspondingly. The above manipulations established that the inward currents recorded in the present study were due to the inward movement of Ba^{++} via Ca^{++} channels.

The bath solution used to record Na^+ and K^+ channel currents was composed of (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1.8, HEPES 10, and glucose 10.

The internal solution used to record Na^+ and K^+ channel currents contained (in mM): KCl 130, EGTA 11, $CaCl_2$ 1, $MgCl_2$ 2, NaCl 10, ATP-Na_2 4, HEPES 10, and glucose 5.

The osmolality of all solutions was adjusted to 310-320 mOsm and the pH to 7.4 using HCl, CsOH, NaOH or $Ba(OH)_2$ as required. All solutions were filtered through a filter with a pore size of 0.22 μm in diameter.

2.2.2. Whole-cell recording.

The Petri dish with the neuroblastoma cells attached was mounted on to the stage of an inverted phase contrast microscope and monitored using a video camera (JVP, GX-5 700). The pipettes were fabricated from borosilicate thin-walled glass tubes (OD 1.2 mm, ID 0.9 mm). They were pulled using a two stage microelectrode puller (Narishige PP-83, Japan) and the tip was fire polished using a microforge (Narishige MF-83, Japan). The resistance of pipettes filled with the pipette solution used for calcium channel current recording was from 2 to 5 megohms. The pipette, which had a small positive internal pressure in order to keep the tip clean, was pushed onto the cell surface using a three dimensional micromanipulator (Narishige, Japan). Suction induced negative pressure was then applied to the pipette. To facilitate the seal between the tip of the pipette and membrane patch, further suction was applied until no additional increase in seal resistance occurred. The seal resistance was usually in the range of 20 - 30 gigaohms. Further suction ruptured the cell membrane under the tip of pipette, and the pipette solution dialysed the cell.

Two indications of free access to the cell cytosol were then observed: 1) A capacitive current appeared which was due to cell capacitance; and 2) a membrane current could be recorded as a function of test potential.

Membrane current recordings were made using the whole cell configuration of the standard patch clamp technique (Hamill *et al.*, 1981). The pipette solution was connected via an Ag/AgCl electrode to an Axopatch-1B (Axon Instruments, Inc., CA) patch clamp amplifier with a 500 megohm feedback resistor in the headstage. The bath solution was held at ground potential via an agar/KCl bridge. All signals were filtered at 1 KHz (4-pole low-pass Bessel filter), analog to digital converted and stored on the floppy disk of a personal computer using a pClamp software (version 5.5.) and a TL-1 DMA interface (Axon Instruments, Inc., CA). In some earlier experiments, VCAN, version 1.2. (software package provided by Dr. J. Dempster, University of Strathclyde, Glasgow) was used to drive the DT2801A interface (Data Translation, Inc).

In order to obtain a good space clamp, cells with a diameter approximately 36 μm or less and neurites less than the soma diameter were chosen (Tsunno *et al.*, 1986). An acceptable temporal and spatial clamp was evident from the following observations: 1) The capacitive current settled fast enough to separate it from the relatively slow calcium currents. 2) The capacitive current recorded under patch clamp conditions had an exponential decline. 3) The activation, inactivation (T-type), and slow inactivation (L-type) segments of inward current at a fixed test pulse did not show any abnormal notches which was a sign of a poor spatial clamp. 4)

The current against voltage (I-V) relationships of predominantly T or L channel currents were smooth and bell-shaped. No abrupt changes of inward current corresponding to the direction of either hyperpolarization or depolarization were observed (Colatsky *et al.*, 1979).

Leakage and capacitive currents were subtracted on line with the P/2 protocol using pClamp software. The subpulse used to subtract the leakage current did not activate any ionic currents within the test pulse range examined. In the presence of 2 mM La^{+++}, the I-V relationship of the leakage current is linear at negative membrane potentials and shows a minor upward deflection at positive membrane potential, for which the intracellular Cs^{+} may be partially responsible.

The accuracy of the voltage clamp over the whole cell membrane is affected by the product of the magnitude of the series resistance and of membrane currents. In case of a large series resistance, there is a deviation of the membrane potential from the command potential. Since in the present study, the peak currents were usually small (approximately 200 pA) with 20 mM Ba^{++} as the charge carrier and the series resistance was usually less than 10 megohms (estimated using Axopatch compensation), the voltage error was less than 2 mV, and hence, series resistance compensation was not usually employed. In cases where the voltage error was greater than 2 mV (Regan *et al.*, 1991), series resistance was compensated electronically using the Axopatch 1-B electronics. If the compensation was not satisfactory, the data were not used. In most cases, pulses of 200 msec duration were applied at intervals of 5 to 10 sec to allow complete recovery of the VDCC from

inactivation.

The decline of calcium channel currents with time ("run-down") is a universal phenomenon in patch-clamp studies. If the current magnitude did not change from the third to the fifth minute after the rupture of the cell membrane, the rate of "run-down" of the inward current was usually negligible for 20 min or longer in N1E-115 cells. Hence, only cells which had stable inward currents from the third to fifth minute after penetration of the membrane were used. If the currents were not stable as described, the calcium channel current decayed too fast to distinguish "run-down" of the current from the inhibitory or excitatory effect of the agents. No calibration, hence, was made for the spontaneous decline of the inward current in measuring the effect of various agents on the calcium channel current.

The I-V plots were constructed using the peak values of the inward current (leakage corrected) from the original traces. At the end of the fifth minute, peak currents of calcium channels were recorded and the I-V relationship was plotted as the control. Different agents were then added directly to the bath solution and the change in the inward current was monitored continuously. One to three min were required for the agents to be distributed evenly throughout the bath solution. Hence, I-V relationships of calcium channels were obtained after at least a 3 min exposure of the cell to each agent to obtain a stable response of the calcium channel currents. When the recorded amplitude of the inward currents was 5% higher or lower than that obtained 30 second before, an increase or decrease, respectively, in the amplitude of the inward current was designated.

In some cells the bath solution was changed by using a perfusion system to washout the agent in order to determine whether the effects of these agents were reversible. The replacement of bath solution was executed for 2 min or more with an exchange rate of approximately 1.4 ml/10 sec. This procedure further excluded the influence of "run-down" of calcium channel currents.

All experiments were carried out at room temperature (21-23°C).

2.3. Spectral analysis.

All absorbance measurements were carried out using an ultraviolet/visible (UV/VIS) Spectrophotometer (Lambda 3A mode, Perkin-Elmer). To measure the absorption spectra 1-cm, quartz cuvettes were filled with different solutions. The absorbance of nifedipine dissolved in different solvents was measured in the wavelength range of 250 to 450 nm in which the absorbance maximums of different nifedipine preparations could be observed. The absorbance maximum of nifedipine preparations occurred at 342 nm. For the same reason, the absorbance of different Bay K-8644 preparations was measured by choosing a wavelength range of 300 to 500 nm. The absorbance maximum of Bay K-8644 preparations occurred at 422 nm. The absorption spectrum of the bath solution, which was used to record calcium channel currents, was taken as the reference. 10 μl of nifedipine stock solution (3 mM to 36 mM) or Bay K-8644 stock solution (0.3 mM to 30 mM) was added to 3 ml bath solution to reach different final concentrations of nifedipine or Bay K-8644 in the bath. The concentration of the solvent was always constant at 0.3%. The absorption

spectra of nifedipine or Bay K-8644 were determined by measuring the sample of the mixture of bath solution and stock solutions of nifedipine or Bay K-8644 dissolved in DMSO, EtOH, acetone or PEG. The reference absorption spectrum was subtracted from all absorption spectra of nifedipine or Bay K-8644. The plot of absorbance against drug concentrations was constructed by using the absorption maximum determined at each concentration of nifedipine or Bay K-8644. Absorbance measurements were conducted at room temperature (21-23°C).

2.4. Drugs.

Nifedipine (Sigma Chemical Co., St. Louis, MO) was dissolved in 99.5% acetone (Fisher Sci. Co., Edmonton, Canada), 98% ethanol (EtOH) (Sigma), 100% PEG (Sigma), or 100% DMSO (Sigma) to make different stock solutions with various concentrations (3 μM to 9 mM). Bay K-8644, i.e. ($\pm$)Bay K-8644, (Calbiochem, Los Angeles, CA) was dissolved in EtOH, DMSO, or PEG to make different stock solutions at concentrations from 0.3 μM to 3 mM, respectively. These stock solutions were always protected from exposure to light since the molecules of DHP contain a photolable structure which can be photochemically inactivated. The DHPs become ineffective as Ca channel agonists or antagonists (Morad *et al.*, 1983; Sanguinetti and Kass, 1984b; Nerbonne *et al.*, 1985). By using stock solutions with different concentrations, different final concentrations of nifedipine or Bay K-8644 in 3 ml of bath were achieved with the addition of a fixed volume of nifedipine or Bay K-8644 stock solution, i.e. 10 μl. Hence, the solvent concentration in the bath was always

0.3%. In order to examine the possible effects of solvents on ionic currents, experiments were also carried out using only the solvents. The effect of nifedipine or Bay K-8644 on calcium channel currents was examined at a single concentration per dish. None of the solvents were allowed to accumulate in the bath.

In some experiments, nifedipine or Bay K-8644 was first dissolved in DMSO at a concentration of 30 mM, and then dried by using a flash evaporator (RV 05 S2 Janke and Kunkel GmbHu. CoKG IKA-WerK T816 Staufen) at 50°C for 24 hr in the dark. The dried nifedipine or Bay K-8644 powder was re-dissolved in different solvents, i.e. ethanol or acetone.

2.5. Statistics.

The data are shown as means ± S.E.M. Tests of significance were performed using the Student's t-test for paired group data and the analysis of variance in conjunction with the Newman-Keul's test for multiple group data. A difference is considered significant when p values are less than 0.05.

CHAPTER 3

Electrophysiological Characterization of Calcium Channel Currents in Neuroblastoma Cells

3.1. The T channel current.

In cells cultured in normal medium, step depolarizations from a holding potential of -80 mV to potentials more positive than -60 mV elicited a transient inward Ba^{++} current. Fig. III-1 shows a series of current records and the associated current-voltage (I-V) relationship in one cell. The time course of this current was characterized by fast activation (8-14 msec) and complete inactivation (half time, 40-70 msec). The inward current was activated at a membrane potential of -60 to -50 mV and the peak inward current occurred at -30 to -10 mV. The apparent reversal potential for this T channel current was between 40 to 60 mV. When the holding potential was shifted to -40 mV, this inward current was significantly inactivated. Under the culture conditions used in the present study, no detectable L channel currents were seen when the holding potential was -40 mV. Furthermore, this transient inward current was not affected by TEA or TTX, but was completely inhibited by 2 mM La^{+++}. The above results indicate that this inward current was

the voltage-dependent T channel current which is similar to the type I current described by Narahashi *et al.* (1987).

3.2. The L channel current.

In experiments in which L (long-lasting) channels were to be studied, the cells were grown and maintained at confluence for 3-4 weeks with the addition of 2% DMSO to the medium (DMEM). Step depolarizations from a holding potential of -40 mV to potentials more positive than -30 mV activated the L channel or type II current (Narahashi *et al.*, 1987) which exhibited the peak magnitude at 10 to 20 mV. This L channel current was sustained in the open state during the 200 msec period of the depolarizing pulse. Increasing the test pulse duration to 400 msec did not reveal any additional inactivation. The apparent reversal potential for L channel currents ranged between 40 and 60 mV. In addition, this inward current was not inhibited by either TEA or TTX, but was very sensitive to La^{+++}. Fig.III-2 shows the original L channel current records (leakage corrected) and the associated I-V relationship in one cell.

Some of the cells which were cultured in media containing 2% DMSO for less than 3 weeks occasionally expressed both types of inward currents. This is also consistent with the results reported by Narahashi *et al.* (1987).

Electrophysiological characterization strongly suggests the existence of two independent populations of calcium channels, i.e. T and L, in N1E-115 cells. The N

channels as well as L channels are the main components of high-voltage activated calcium channels. Although N channels have been observed in sympathetic neurons (Miller *et al.*, 1988), this specific channel type does not appear to be present in N1E-115 cells, a sympathetic neuron tumor line (Audesirk *et al.*, 1990; Ogata and Narahashi, 1990). There are clear and definitive differences between normal and sympathetic neurons obtained from a tumor. Among examples of these differences is the absence and presence of T channels in normal sympathetic neurons and N1E-115 cells, respectively. There is no doubt that N channels show some resemblance to L channels. Both of them are activated at more positive membrane potentials and can be blocked by ω-conotoxin. However, the L channel currents recorded in my study did not inactivate appreciably and were very sensitive to DHPs (shown in the following chapters). These characteristics of L channels convincingly distinguish them from N channels. Since T and L channels appear in the different stages of differentiation in N1E-115 cells, the expression of N channels in N1E-115 cells, due perhaps to the presence of some specific simulators or the different degree of maturation of cells, is possible.

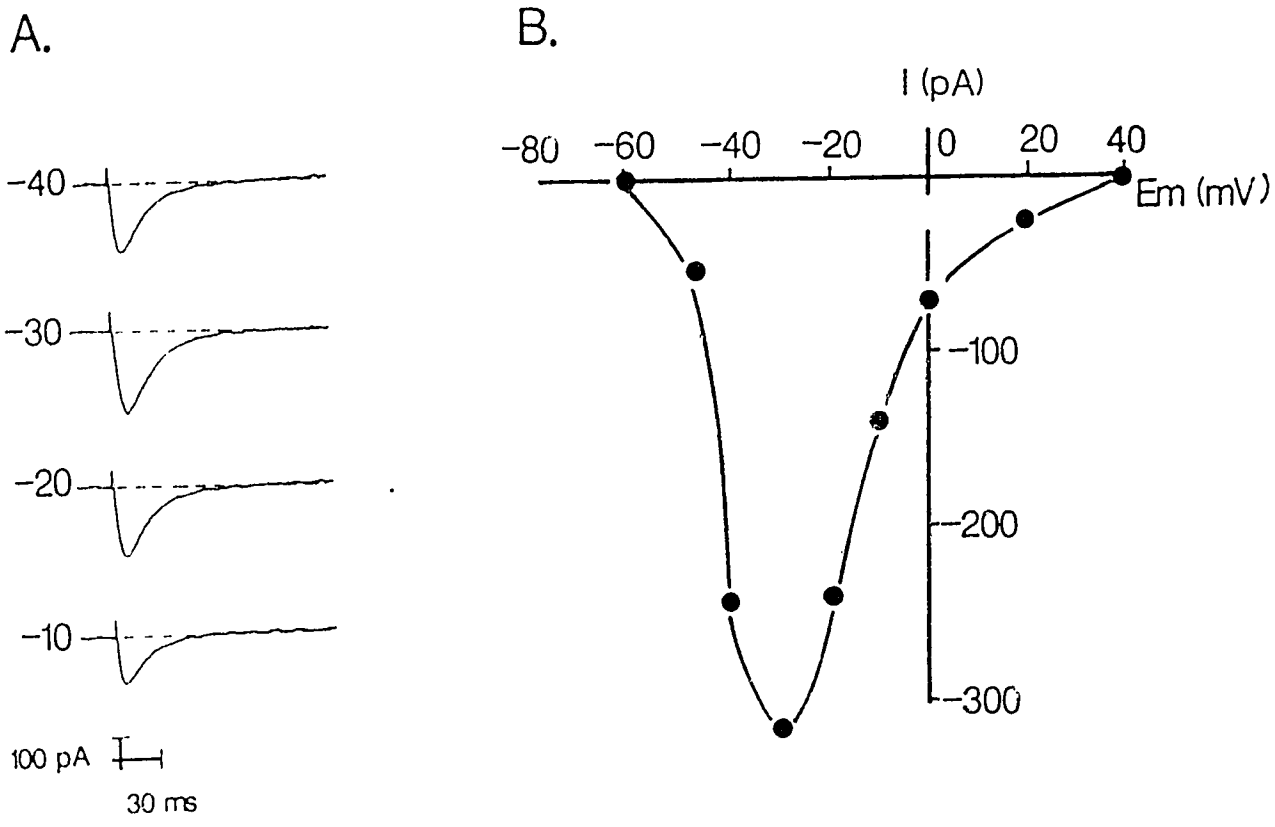
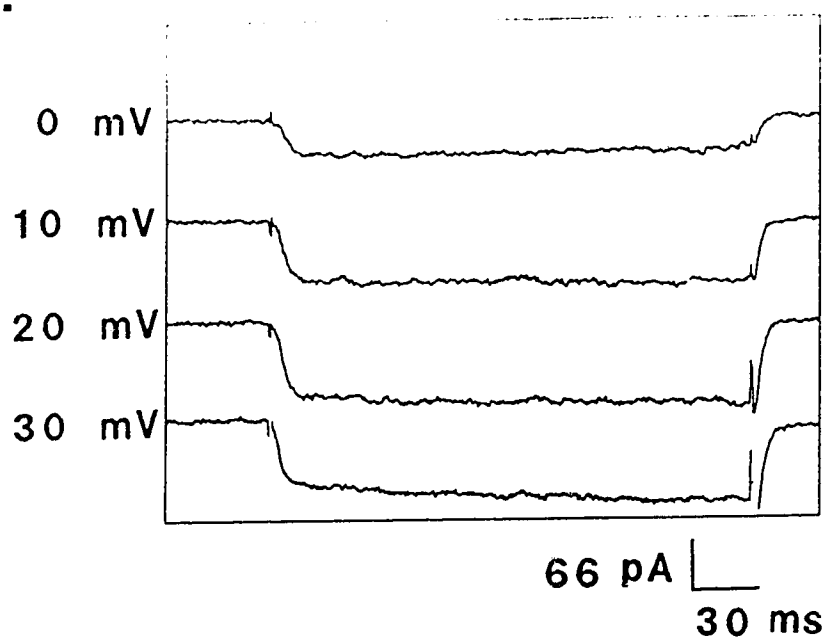

A.
-40
-30
-20
-10
100 pA
30 ms
B.
I (pA)
-80
-60
-40
-20
0
20
40
Em (mV)
-100
-200
-300

Fig.III-1. T channel current records and associated current-voltage relationship in one N1E-115 cell. Holding potential was set at -80 mV. Leakage and capacity currents have been subtracted. **A.** The original current records with the magnitude of the test pulses indicated in mV beside each trace. Zero current levels are indicated by the dashed lines. **B.** The I-V relationship of T channel currents in the same cell as in **A.** The original current traces were recorded using software VCAN (software package provided by Dr. J. Dempster, University of Strathclyde, Glasgow).

A.



B.

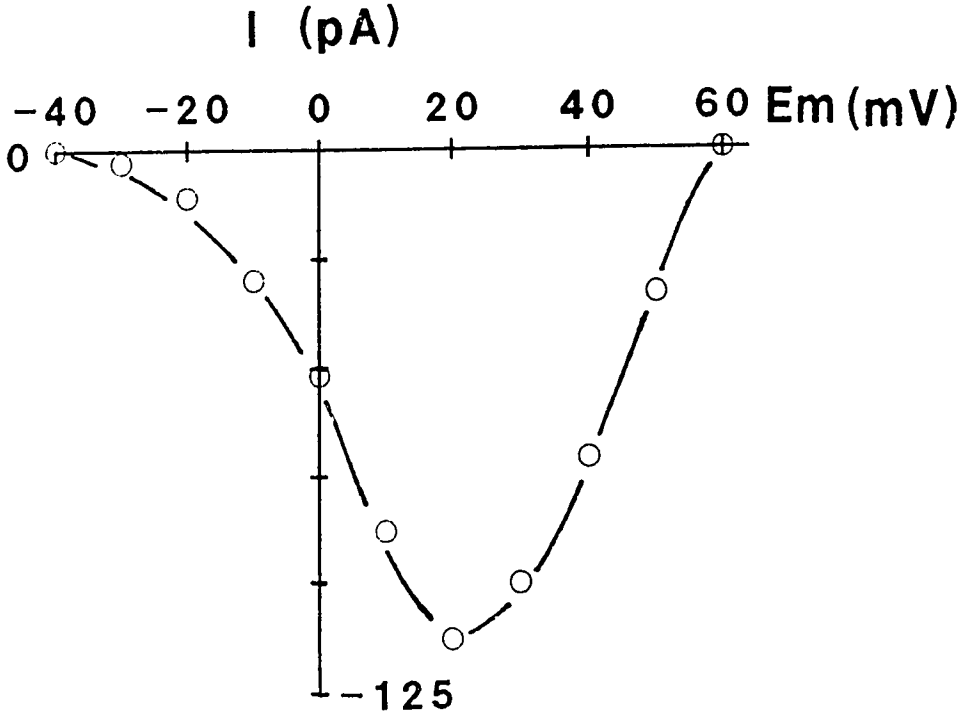


Fig.III-2. L channel current records and associated current-voltage relationship in one N1E-115 cell. Holding potential was set at -40 mV. Leakage and capacitive currents have been subtracted. **A.** The original current records with the magnitude of the test pulses indicated in mV beside each trace. Zero current levels are indicated by the dashed lines. **B.** The I-V relationship of L channels in the same cell as in **A.** The original current traces were recorded using software pClamp (Axon Instruments, Inc., CA).

CHAPTER 4

The Effect of Nifedipine on Calcium Channel Currents

4.1. The effect of nifedipine dissolved in DMSO (nifedipine/DMSO) on the T channel current.

In Fig.IV-1, nifedipine/DMSO at a concentration of 10 μM decreased the T current (45%) in one cell. The activation potential and the apparent reversal potential were not changed by nifedipine/DMSO. The amplitude of T channel currents fully recovered after a 5-min washout with nifedipine/DMSO-free bath solution, indicating that the inhibitory effect of nifedipine/DMSO on the T channel current is reversible. The reversibility of the inhibitory effect of nifedipine/DMSO on T channel currents by the washout protocol eliminated the possibility that the inhibitory effect was due to the deterioration or run-down of T channel currents.

Fig.IV-2 shows the dose-response of T channel currents to nifedipine/DMSO. Nifedipine/DMSO (30 μM) decreased T channel currents to 31 $\pm$ 4% of the control level in 6 cells ($p<0.05$). The amplitude of T currents was reduced to 50 $\pm$ 12% of the control value by 10 μM nifedipine/DMSO in 7 cells ($p<0.05$). At a concentration of 1 μM, nifedipine/DMSO inhibited the T current by 27 $\pm$ 8% in 6

cells ($p<0.05$). The DMSO concentration in the bath was always fixed at 0.3% for each concentration of nifedipine.

The steady-state inactivation curves for the T channel currents before or during exposure to nifedipine/DMSO are shown in Fig.IV-3. The test pulse (duration, 200 msec) to -20 mV was preceded by a 2-sec conditioning pulse beginning at -90 mV and continuing to -20 mV in 10 mV increments. The peak current amplitudes during the test pulse were measured at each conditioning pulse and normalized to the current amplitude obtained at the most negative conditioning pulse. The absolute amplitudes of T channel currents at various holding potentials were decreased by nifedipine/DMSO (Fig.IV-3A). The steady-state inactivation curve in the presence of nifedipine/DMSO was shifted by approximately 10 mV towards more negative potentials. Similar results were obtained in 4 other experiments. There was no significant change in the slope of the normalized steady-state inactivation curve, shown in Fig. IV-3B. These results indicate that nifedipine/DMSO preferentially blocked the T channel in the inactivated state but had no effect on the voltage-dependent inactivation of T channel currents.

The DHP effect on T channel currents may be influenced by the functional state of the calcium channels since some DHPs prefer to bind to the closed state, and some DHPs bind to both open and closed states, of calcium channels (Hill, 1977; Hering *et al.*, 1988; Kamp *et al.*, 1989). Using a concentration-jump technique (step change in concentration), Hering *et al.* (1988) was able to apply drugs to the cell in a time scale of less than 10 msec. Nifedipine was effective in blocking calcium

channel currents only when it was not applied during the sustained current component. The observation by Hering *et al.* (1988) indicated that nifedipine bound to the closed, available state and the inactivated state of calcium channels. To examine the state-dependence of the effect of nifedipine/DMSO on T channel currents, the following experiments were executed as shown in Fig. IV-4. The holding potential was set at -80 mV and a depolarizing test pulse of 200 msec to -20 mV was applied every 15 seconds. This constant depolarizing frequency and duration was maintained before and after the application of nifedipine/DMSO to the bath. Under these conditions, 100 μM nifedipine/DMSO caused a rapid decrease in the amplitude of the T current and completely eliminated the T current 1 minute after addition to the bath solution. Fig. IV-4A-a shows the result from one cell. Similar results were obtained in a total of 6 cells. In another 5 cells, nifedipine/DMSO at a final concentration of 10 μM decreased the T current by approximately 50% (Fig.IV-4B-a). With repeated stimulation, the probability that T channels stay in the open state will be increased. Hence, the above results suggest that nifedipine/DMSO may bind to the open state of T channels. The next set of experiments was designed to address the issue of whether nifedipine/DMSO binds to the closed state of T channels. In this group of experiments, the cell membrane was not depolarized for a period of 2 min after nifedipine/DMSO (100 μM) was added to the bath solution. The first depolarizing pulse following the two minute "quiescent" period revealed complete inhibition of T channel currents (Fig.IV-4A-b). In another cell shown in Fig.IV-4B-b, 10 μM nifedipine/DMSO was applied. After a four minute pulse free

period, the decrease in the amplitude of T channel currents was recorded at the first depolarization and was maintained at this suppressed level. It appears that depolarizing pulses were not a prerequisite for the onset of action of nifedipine/DMSO on T channel currents. Hence, nifedipine/DMSO can bind to the closed state of the T channels to effectively inhibit this channel. The present data, of course, do not exclude the possibility that nifedipine/DMSO may bind to the open and/or inactivated state of T channels as well.

4.2. The effect of nifedipine dissolved in acetone (nifedipine/acetone) or nifedipine dissolved in ethanol (nifedipine/EtOH) on the T channel current.

Since acetone (Cheng and Townley 1983; Pang *et al.* 1990; Wang *et al.* 1990) and ethanol (Almers *et al.* 1981; Arreola *et al.* 1987; Fox *et al.* 1987) have been used to dissolve nifedipine, it is necessary to examine the effects of nifedipine/acetone and nifedipine/EtOH on the T channel current. These effects were compared with the inhibitory effect of nifedipine/DMSO on the T channel current. This is shown in Fig.IV-5. Nifedipine/acetone at concentrations from 1 to 100 μM had no effect on the amplitude, kinetics or I-V relationship of the T channel current. The current required to maintain the cell at the holding potential and leakage current were also the same before and after the application of nifedipine/acetone. Similar results have been reported by Wang *et al.* (1990). In addition, nifedipine/EtOH within the concentration range of 1 to 100 μM did not change the amplitude or kinetics of T channel currents (Fig.IV-6).

4.3. The effect of nifedipine dissolved in PEG (nifedipine/PEG) on the T channel current.

The sensitivity of the transient inward current to nifedipine dissolved in PEG is shown in Fig.IV-7. In this cell, nifedipine/PEG at a concentration of 10 μM decreased the T channel current by 32%. Similar inhibition of T channel currents by nifedipine/PEG was also observed in 3 other preparations. The activation potential and the apparent reversal potential of T channel currents were not affected by nifedipine/PEG.

4.4. The effect of solvents on the T channel currents.

To demonstrate whether solvents alone could affect the T channel current, DMSO, PEG, acetone or ethanol were added to the bath solution to obtain a final concentration of 0.3%. In 4 experiments, the final concentration of DMSO was raised to 0.5% (Fig.IV-8). The incubation time with DMSO, acetone or ethanol was 5 to 30 min in all cells tested. Treatment of the cells with DMSO (0.3% or 0.5%), acetone (0.3%), EtOH (0.3%) or PEG (0.3%) did not change the amplitude or kinetics of the T channel currents. The current-voltage relationship of the T channel current was also unchanged.

Inhibition of the T channel current was observed when nifedipine/DMSO (30 μM) was applied after the cells were pretreated with 0.3% acetone for 10 min (Fig.IV-9). There was no significant difference in the inhibition of T channel

currents by nifedipine/DMSO with or without pretreatment of the cells with 0.3% acetone (n=4, $p>0.05$). In addition, after a 10 min pretreatment of the cells with DMSO, nifedipine/acetone (30 μM) failed to modify the T channel current, as shown in Fig.IV-10 (n=4, $p>0.05$). These experiments ruled out the possibility that the solvents affect channel protein, and hence, the binding of nifedipine to T channels.

4.5. The interaction of nifedipine/DMSO with nifedipine/EtOH or nifedipine/acetone.

In order to determine whether nifedipine/DMSO and nifedipine/acetone or nifedipine/EtOH act on the same site(s) on or near T-type calcium channels, the inhibitory effect of nifedipine/DMSO on the T channel current in the presence of nifedipine/acetone (n=7) or nifedipine/EtOH (n=4) was tested. Nifedipine/acetone (30 μM) was first added to the bath solution. No change in either the amplitude or kinetics of the T channel current could be detected. Five minutes later, nifedipine/DMSO (1 μM) was applied to the same cell. The T channel current was significantly inhibited after the addition of nifedipine/DMSO (Fig.IV-5). This inhibition could also be reversed by the washout protocol. The effect of nifedipine/DMSO on the T channel current in the presence or absence of nifedipine/acetone was not significantly different except that the time for inhibition of the T channel current by nifedipine/DMSO was delayed by 1-2 min in the presence of nifedipine/acetone. In addition, the inhibitory effect of 1 μM nifedipine/DMSO was not changed in the presence of 30 μM nifedipine/EtOH

(Fig.IV-6). It appears that the actions of nifedipine/DMSO and nifedipine/acetone or nifedipine/EtOH on the T channel were not competitive within the concentration range studied. It is important to note that in order to exclude the possible interaction of nifedipine/DMSO and nifedipine/acetone on T channels the inhibition of T channel currents by nifedipine/DMSO should be examined over the effective concentration range of nifedipine/acetone (i.e. complete dose-response).

4.6. The effect of dried nifedipine/DMSO redissolved in acetone or ethanol on the T channel current .

The next group of experiments was carried out in order to determine whether DMSO modifies the conformation of nifedipine. Nifedipine was first dissolved in DMSO at a concentration of 30 mM and, then, dried using a flash evaporator at 50 °C for 24 hr in the dark. The dried nifedipine/DMSO powder was then re-dissolved in DMSO (nifedipine/D-DMSO), acetone (nifedipine/D-acetone), or ethanol (nifedipine/D-EtOH). Fig.IV-11.shows that nifedipine/D-EtOH significantly inhibited the T channel current. The maximum suppression of the T channel current usually occurred at about 10 to 15 min after the drug was added, and disappeared after 5 min of washout with drug-free solution. This indicates that the decrease in the T channel current in the presence of nifedipine/D-EtOH was not due to the "run-down " or deterioration of currents. Compared to the onset time of the maximum effect of nifedipine/DMSO, there was approximately a 5 to 10 min delay in the onset of the effect of nifedipine/D-acetone or nifedipine/D-EtOH on the T

channel current in all experiments ($n=5$, $p<0.05$). It is suggested that acetone or ethanol may also exert some inhibitory effects on the action of nifedipine.

Nifedipine/D-DMSO decreased T channel currents by 55 ± 8% at a concentration of 10 μM in 5 cells (Fig.IV-12). There was no significant difference in the inhibitory effect and the onset time of the inhibition of T channel currents when comparing 10 μM nifedipine/DMSO and nifedipine/D-DMSO ($p>0.05$). Fig.IV-12 shows that at a concentration of 10 μM, nifedipine/D-acetone inhibited the T channel current by 33 ± 12% ($n=6$, $p<0.05$), and nifedipine/D-EtOH by 37 ± 10% of control level ($n=6$, $p<$ [illegible]. These results demonstrate that the molecular conformation of nifedipine [illegible] modified by DMSO so that subsequent preparations of nifedipine dissolved in acetone or ethanol inhibited T channel currents in N1E-115 cells.

4.7. The solubility of nifedipine dissolved in different solvents.

Since nifedipine is insoluble in water, the different effects of nifedipine/DMSO and nifedipine/acetone or nifedipine/EtOH on T channel currents might be due to the precipitation of nifedipine from different solvent preparations when the drug stock solution was added to the bath. If this is the case, the effect of nifedipine/DMSO on T channel currents will be only a result of less precipitation of nifedipine with DMSO as the solvent. However, data from the present studies disagree with this hypothesis. Nifedipine at concentrations from 0.3 μM to 36 mM in the stock solutions was completely soluble in DMSO, acetone, or ethanol. With

a fixed solvent concentration in the bath (0.3%), different final drug concentrations were obtained by adding nifedipine stock solutions to the bath solution. Under these conditions, ultraviolet spectral analysis showed that the absorbance maximum of nifedipine at different concentrations (10 to 120 μM) in the bath varied, but always occurred at the same wavelength, i.e., 342 nm (Fig.IV-13-A). The varied absorbance maxima of nifedipine/acetone or nifedipine/EtOH at different concentrations also occurred at 342 nm (Fig.IV-13-B). The relationship of maximum absorbance spectra and nifedipine concentrations is shown in Fig.IV-14. The absorbance maximum of nifedipine dissolved in DMSO, acetone, or ethanol rose proportionally with increasing concentrations. There was no significant difference in the absorbance maximum of nifedipine dissolved in the three solvents at different concentrations. The linear plot of absorbance against nifedipine concentrations (Fig.IV-14) indicates that the precipitation of nifedipine in bath solution under the present conditions was unlikely.

4.8. The effect of nifedipine dissolved in different solvents on the L channel current.

L channel or type II currents in N1E-115 cells (Narahashi *et al.* 1987) were activated at -30 mV from a holding potential of -40 mV, but did not inactivate during 200 msec depolarization. Nifedipine/DMSO (1 μM) reduced L channel currents to 25% of the control level, as shown in Fig.IV-15.

Fig.IV-16 shows the dose-response of the L channel current to nifedipine

dissolved in either DMSO or acetone. At concentrations from 10 nM to 1 μM, nifedipine/acetone had no significant effect on L channel currents. At concentrations higher than 10 μM, nifedipine/acetone showed an inhibitory effect on the L channel currents in N1E-115 cells ($p < 0.05$), which was consistent with the previous report from this laboratory (Wang *et al.* 1990). However, within the same concentration range (Fig.IV-16), nifedipine/DMSO more effectively inhibited the L channel current in neuroblastoma cells. The dose for half-maximal inhibition of L channel currents by nifedipine/DMSO is approximately 100 nM. The solvent concentration in the bath for each dose of nifedipine was always fixed at 0.3%.

The effects of nifedipine/PEG and nifedipine/EtOH on L channel currents were also examined. Nifedipine/PEG at a concentration of 100 μM decreased the L channel current by 46% of the control level ($n=4$, $p < 0.05$). Furthermore, L channel currents were inhibited by 50% using 100 μM nifedipine/EtOH ($n= 3$, $p < 0.05$). Compared with the effect of nifedipine/DMSO, at the same concentration nifedipine/PEG and nifedipine/EtOH showed a smaller inhibitory effect on the L channel current in N1E-115 cells. However, there was no significant difference in the inhibition of L channel currents induced by nifedipine/acetone, nifedipine/PEG or nifedipine/EtOH ($p > 0.05$).

4.9. The effect of solvents on the L channel current.

The amplitude and the kinetics of L channel currents were not affected by DMSO, acetone, PEG, or ethanol at the concentration of 0.3% (v/v) in the bath

solution (n=5, P>0.05). The current-voltage relationship of the L channel current was also unchanged in the presence of these solvents. Fig.IV-17 shows that DMSO, even at higher concentration (0.5%, v/v) than that used in the present studies, did not affect the L channel current. Similar results were also observed in 4 other cell preparations.

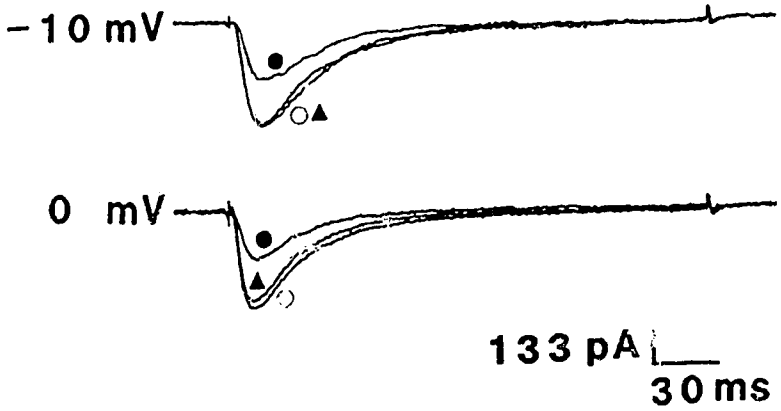
4.10. The effect of nifedipine/DMSO on Na^+ and K^+ channel currents.

The recording of sodium and potassium channel currents was conducted using N1E-115 cells which were grown in normal media. A transient inward current and an outward current were recorded in N1E-115 cells. The inward current was elicited at -50 mV from the holding potential of -80 mV, reached a peak at -10 to -20 mV, and reversed at 50 to 60 mV. This transient inward current was characterized by fast activation (1-1.5 msec) and inactivation (half time, 2-3 msec). Pharmacological identification of this transient inward current as the sodium channel current was carried out using a specific sodium channel blocker, tetrodotoxin (TTX) (Narahashi, 1974). When TTX was included in the bath solution at the concentration of 1 or 10 μM, the transient inward current was completely eliminated. The replacement of Na with Tris in the bath solution also eliminated this inward current. There was no long-lasting inward current component under the present recording conditions. This indicates that the inward current was the Na^+ channel current. Although 1.8 mM Ca was included in the bath solution, neither T nor L type calcium channel currents were detectable. The outward current was inhibited by TEA. This outward current

was not completely abolished by 20 mM TEA in the bath solution. When the K^+-free pipette solution with a high concentration of Cs^+ was used, the residual outward current was abolished. These observations suggest that the outward current, under the present experimental conditions, was composed of at least two types of K^+ channel currents (Fig.IV-18). One was TEA sensitive and another was TEA insensitive. Fishman *et al.* (1981) also described similar potassium channel currents in N1E-115 cells.

The specificity of the nifedipine/DMSO effect on voltage-dependent calcium channels was further investigated since it may be questioned that nifedipine/DMSO affects all ionic channels non-specifically . In 4 N1E-115 cells, nifedipine/DMSO at a concentration of 10 μM did not change the amplitude ($p > 0.05$), the I-V relationship or the kinetics of the Na^+ and the K^+ channel currents.

A.


-10 mV
0 mV
133 pA
30 ms

B.

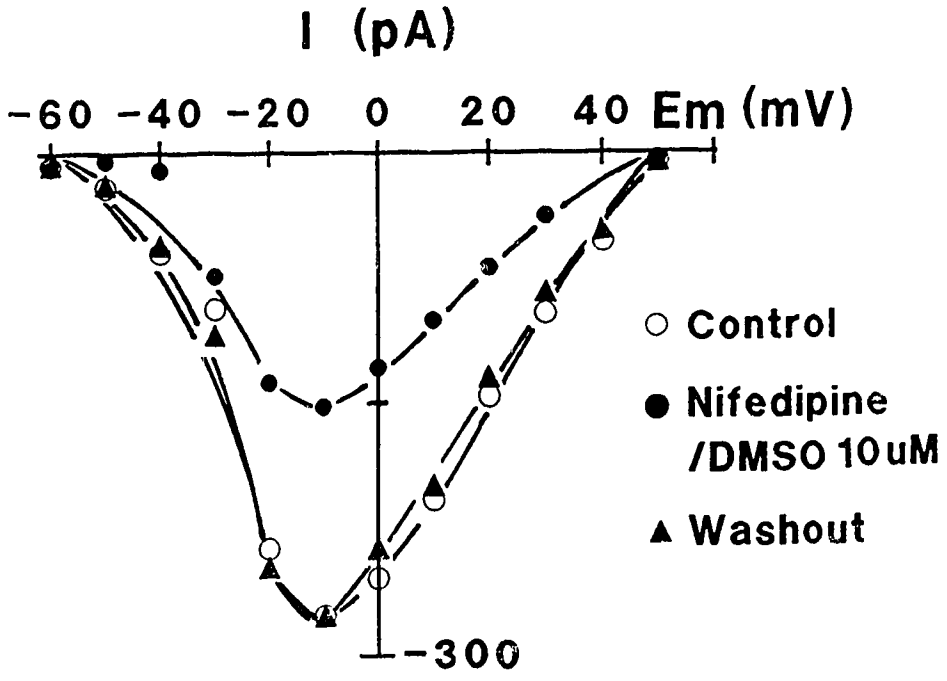

I (pA)
-60 -40 -20 0 20 40 Em (mV)
-300
Control
Nifedipine
/DMSO 10uM
Washout

Fig.IV-1. The effect of nifedipine/DMSO on T channel currents in one N1E-115 cell. **A.** original traces of T channel currents recorded before (open circles), 5 min after exposure to 10 μM nifedipine/DMSO (filled circles), and following 5 min of washout with drug-free bath solution (filled triangles). Leakage and capacity currents have been subtracted from the traces shown. Holding potential was set at -80 mV. **B.** The I-V relationship of T channel currents were obtained under different conditions as described above. The T current was activated at -50 mV, peaked at -10 mV and reversed its direction at approximately 50 mV.

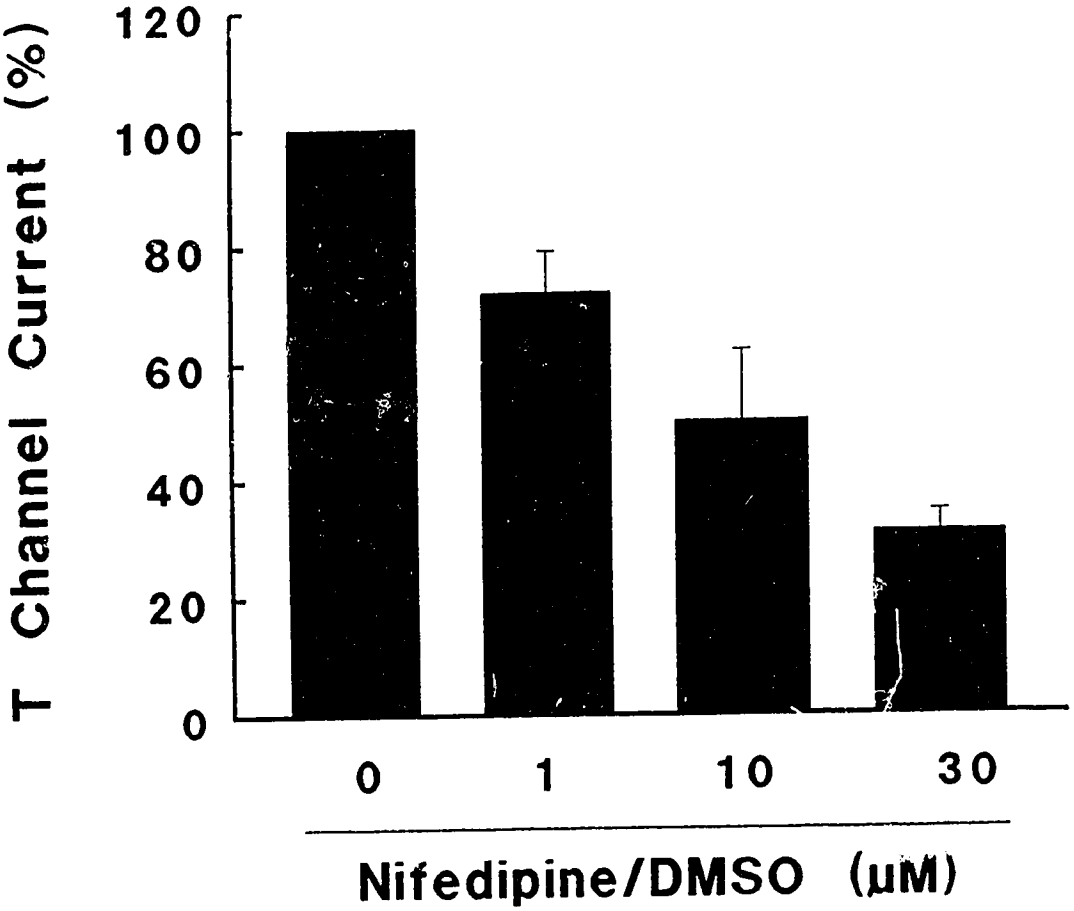

T Channel Current (%)
120
100
80
60
40
20
0
0
1
10
30
Nifedipine/DMSO (μM)

Fig.IV-2. The concentration-dependent inhibition of T channel currents by nifedipine/DMSO in N1E-115 cells. At each concentration, 6 to 7 cells were examined. The peak T channel currents in the absence of nifedipine/DMSO was taken as 100%. The final concentration of DMSO in the bath (0.3%) was the same at each concentration of nifedipine. Bars represent the SEM.

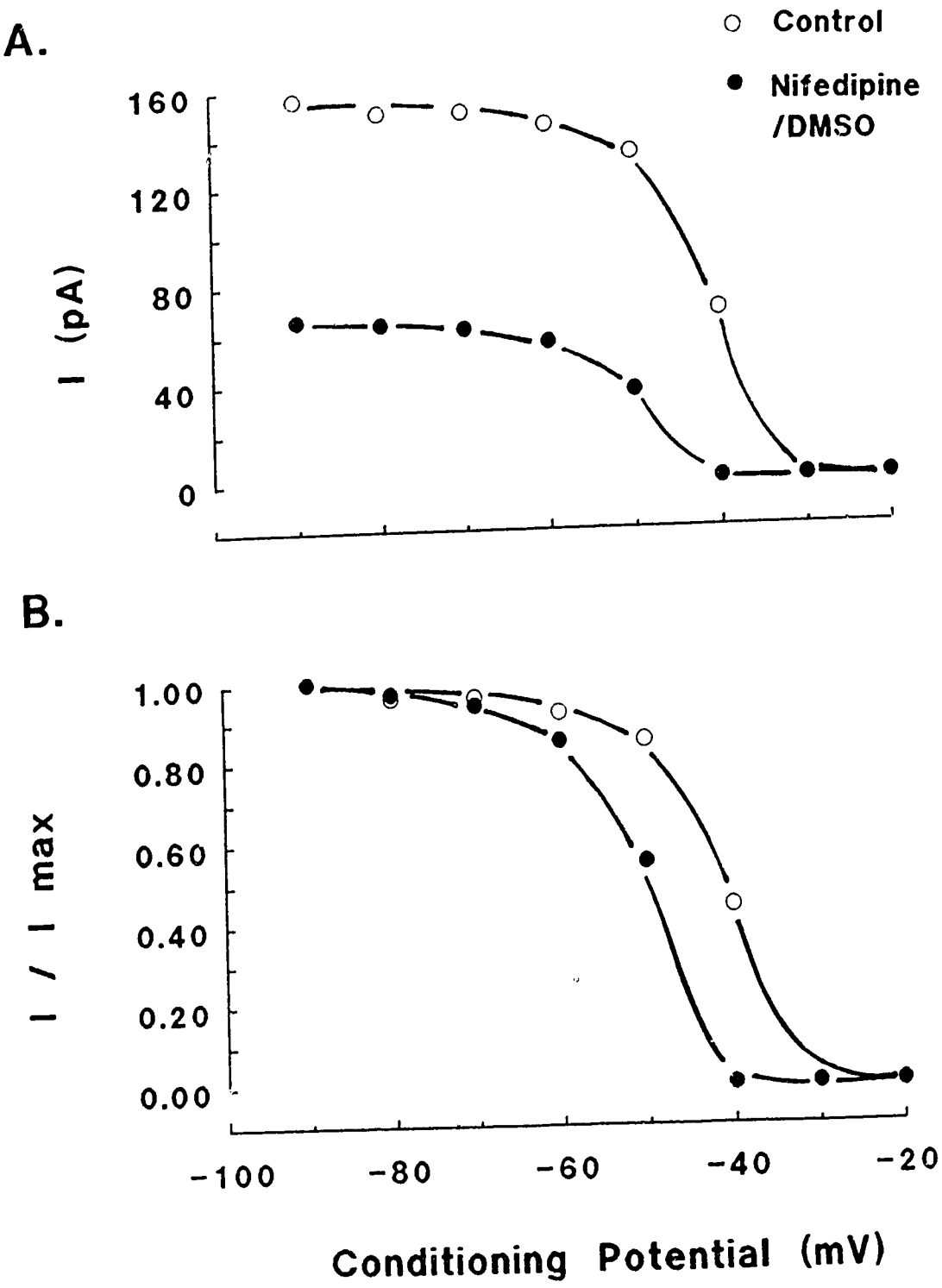

A.
Control
Nifedipine
/DMSO
160
120
80
40
0
I (pA)
B.
1.00
0.80
0.60
0.40
0.20
0.00
I / I max
-100
-80
-60
-40
-20
Conditioning Potential (mV)

Fig.IV-3. The steady-state inactivation of T channel currents in the absence or presence of 10 μM nifedipine/DMSO. The conditioning potential was changed in a stepwise manner with a fixed membrane depolarization to -20 mV. The membrane was held for 2 sec at each conditioning potential. **A.** The absolute steady-state inactivation curve for T channels was suppressed by nifedipine/DMSO. **B.** The normalized steady-state inactivation curve for T channels was shifted towards more negative potentials by 10 mV. The smooth curves in **A** and **B** were fitted by eye. The membrane potential at which one-half of T channels were inactivated was -40 mV and -50 mV in the absence and then presence of nifedipine/DMSO, respectively.

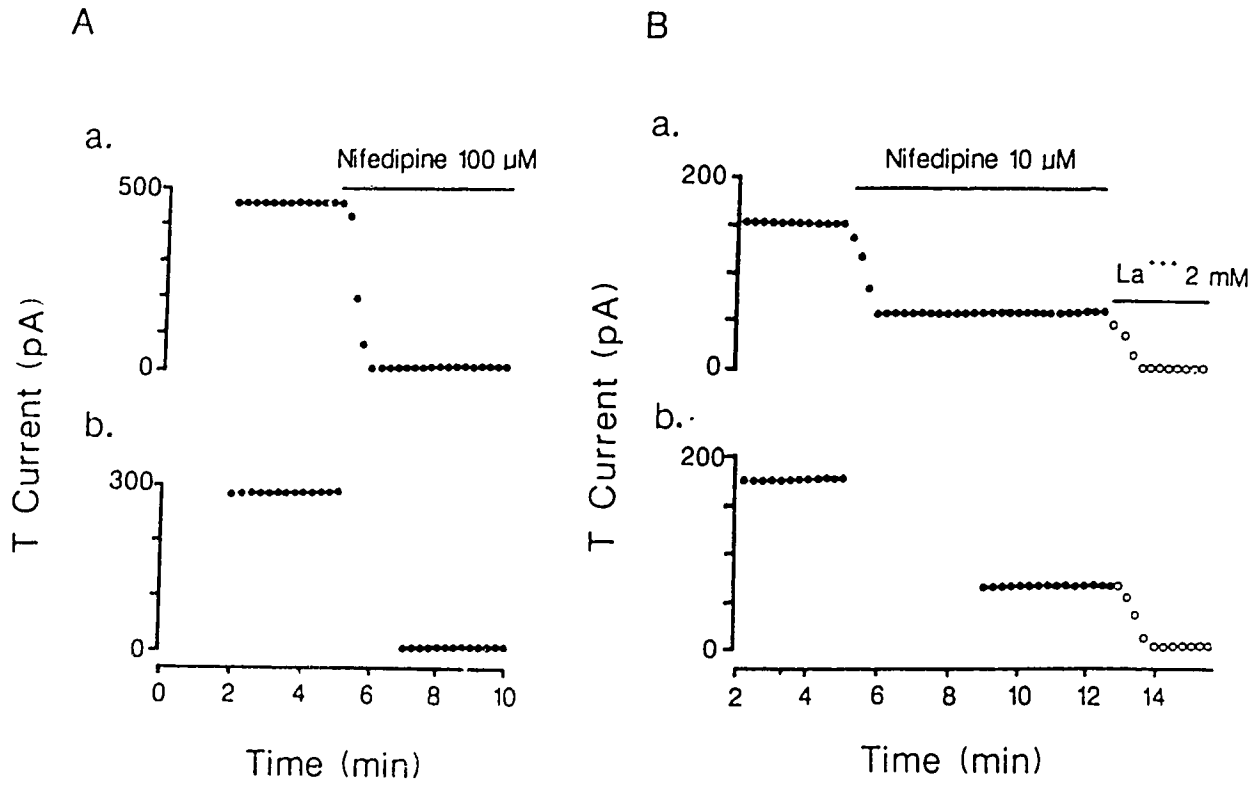

A
a.
Nifedipine 100 µM
500
0
b.
300
0
0 2 4 6 8 10
Time (min)
T Current (pA)
B
a.
Nifedipine 10 µM
200
0
La 2 mM
b.
200
0
2 4 6 8 10 12 14
Time (min)
T Current (pA)

Fig.IV-4. Onset of the action of nifedipine/DMSO on T channel currents in N1E-115 cells. The concentrations of nifedipine/DMSO were 100 μM in **A** and 10 μM in **B**, respectively. Peak inward currents elicited at a membrane potential of -20 mV were measured every 16 seconds (holding potential, -80 mV) in **A-a** and **B-a**. However, the results shown in **A-b** and **B-b** were obtained with a pulse-free period of 2 and 4 min, respectively. The first depolarizing pulse after the administration of nifedipine/DMSO revealed the inhibition of T channel currents by 100% in **A-b** and 63% in **B-b**. There was no further change in the decrease of T channel currents by sequentially repeated pulses with 15 second intervals. Subsequent application of 2 mM La^{+++} completely eliminated the residual T channel currents, shown in **B-b**.

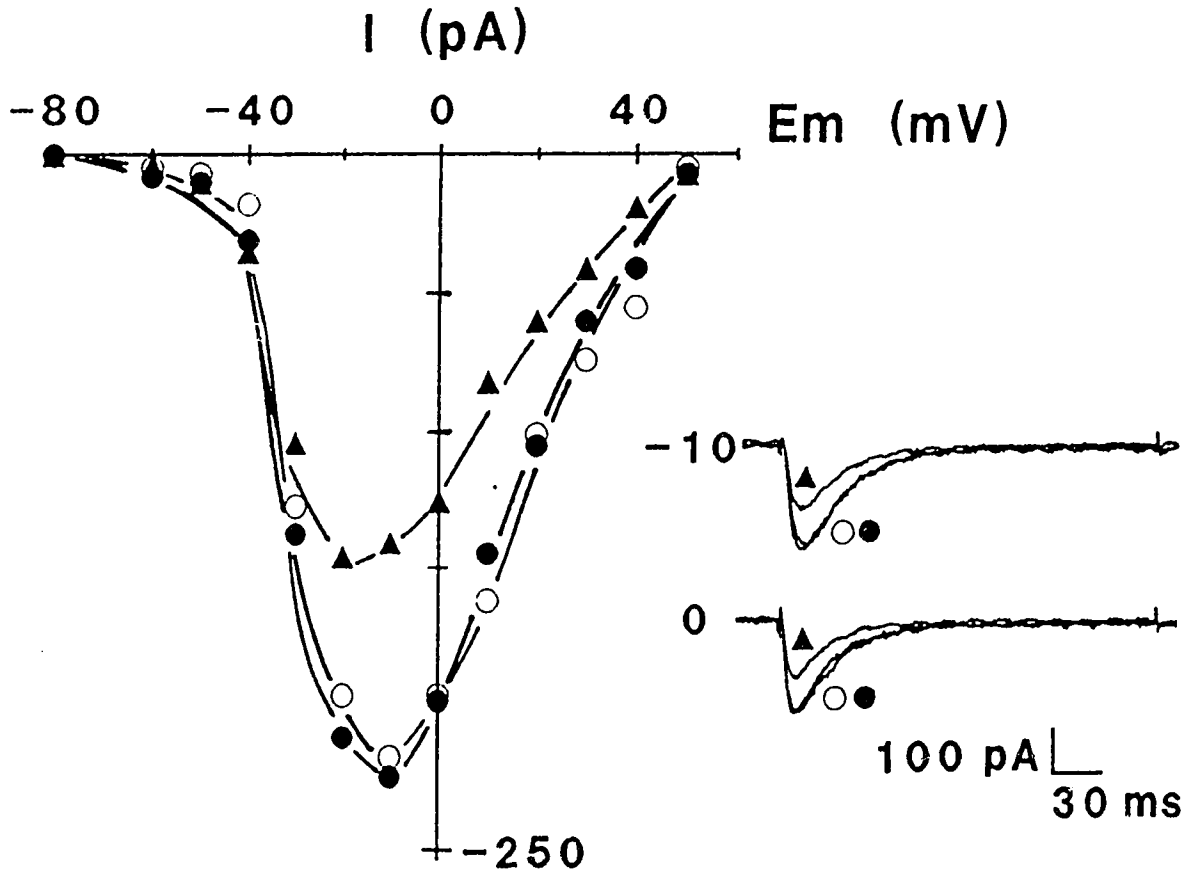

I (pA)
-80
-40
0
40
Em (mV)
-250
-10
0
100 pA
30 ms

Fig.IV-5. The effect of nifedipine/DMSO on T channel currents after pretreatment of the cells with nifedipine/acetone in one N1E-115 cell. Holding potential was -80 mV. Leakage and capacitive currents have been subtracted from the original traces. Control currents were obtained in the absence of any drugs (open circles). Then, nifedipine/acetone (30 μM) was added to the bath (filled circles). Subsequently, nifedipine/DMSO (1 μM) was applied to the same cell (filled triangles). Two sets of original current traces are also shown with the magnitude of the test pulses indicated in mV.

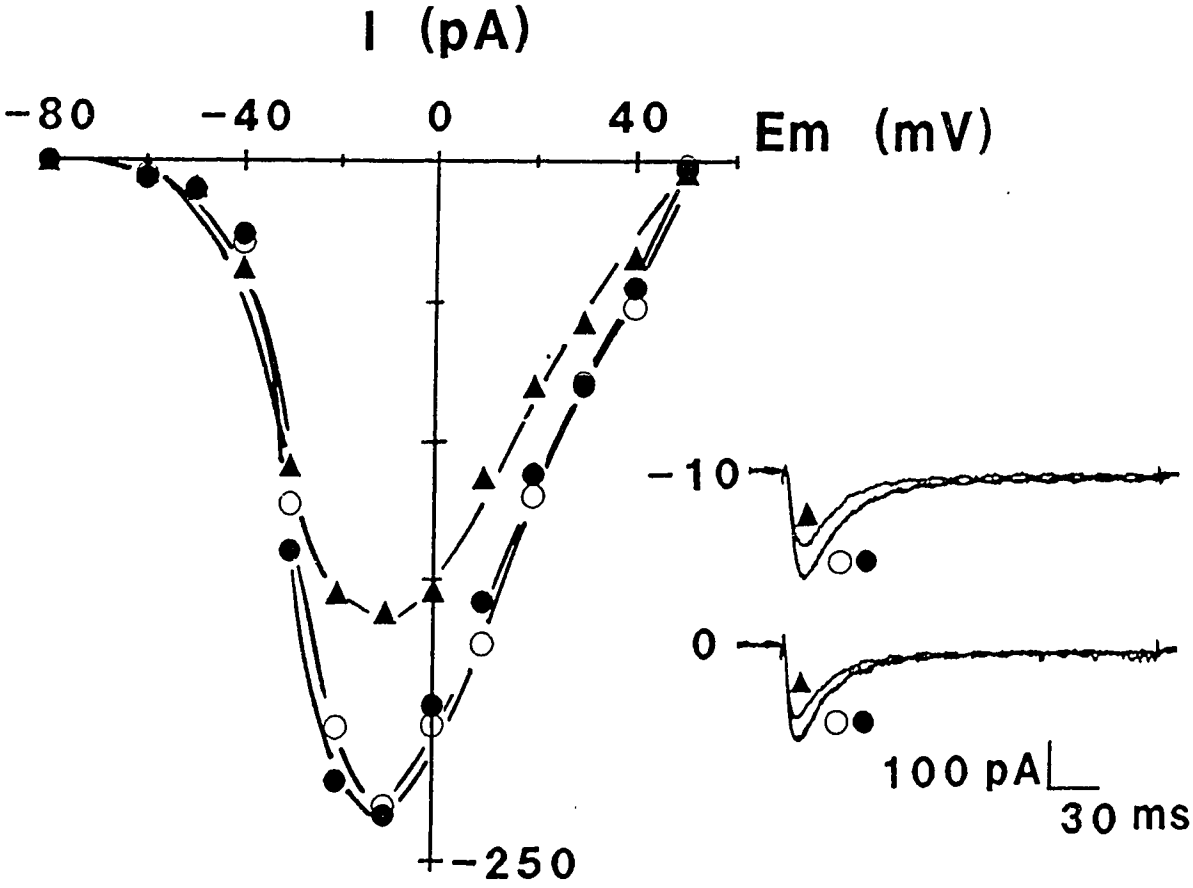

I (pA)
-80
-40
0
40
Em (mV)
-250
-10
0
100 pA
30 ms

Fig.IV-6. The effect of nifedipine/DMSO on T channel currents after pretreatment of the cells with nifedipine/EtOH. The T channel currents were recorded in the same cell in the following sequence: control (open circles), 5 min after the addition of nifedipine/EtOH (30 μM) in the bath (filled circles), and 5 min after the addition of nifedipine/DMSO (1 μM) in the bath (filled triangles). Leakage and capacitive currents were subtracted from the original traces. Holding potential was set at -80 mV. Two sets of original current traces are also shown. The magnitude of the test pulses is indicated in mV beside each set of traces.

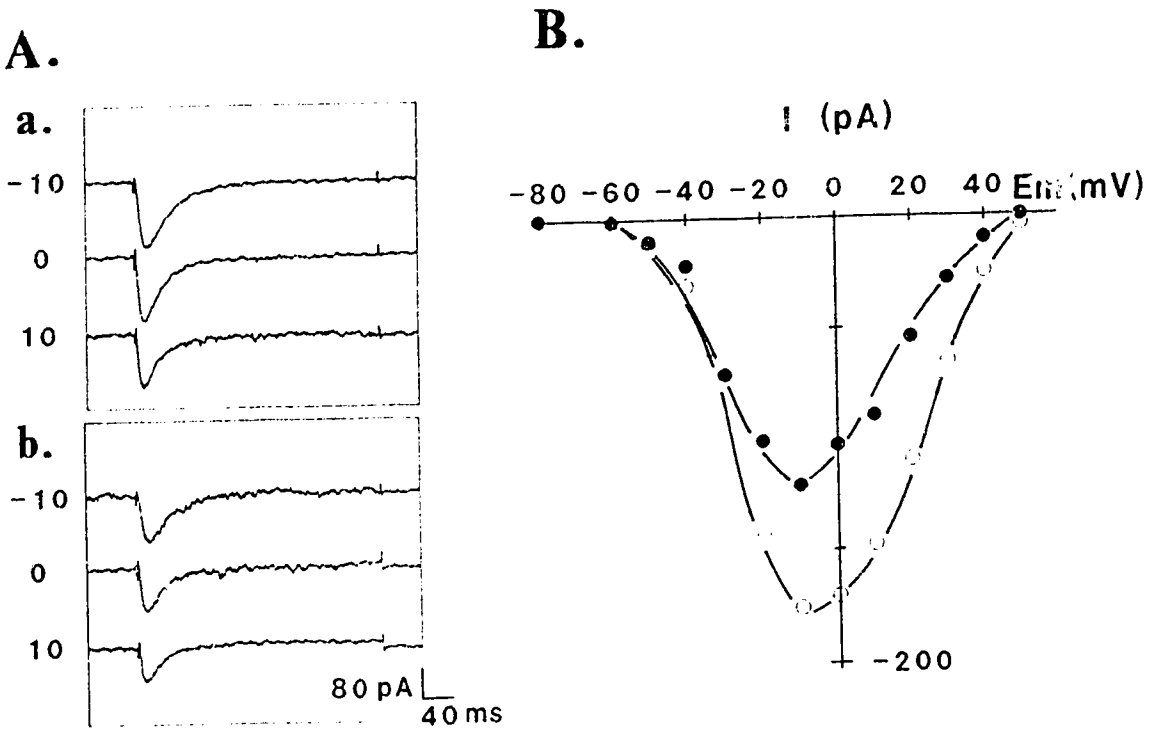

A.
a.
-10
0
10
b.
-10
0
10
80 pA
40 ms
B.
I (pA)
-80 -60 -40 -20 0 20 40 Em (mV)
-200

Fig.IV-7. The effect of nifedipine/PEG on the T channel current in one N1E-115 cell. **A.** The original traces of T channel currents were recorded prior to addition of any drug (shown in a). Nifedipine/PEG (10 μM) was then added to the bath solution (shown in b). The magnitude of the test pulses is indicated in mV beside each trace. Leakage and capacitive currents were subtracted. Holding potential was set at -80 mV. **B.** After the control recording of the I-V relationship was made (open circles), nifedipine/PEG (10 μM) (filled circles) was added to the bath solution. Nifedipine/PEG produced a significant decrease in T channel currents.

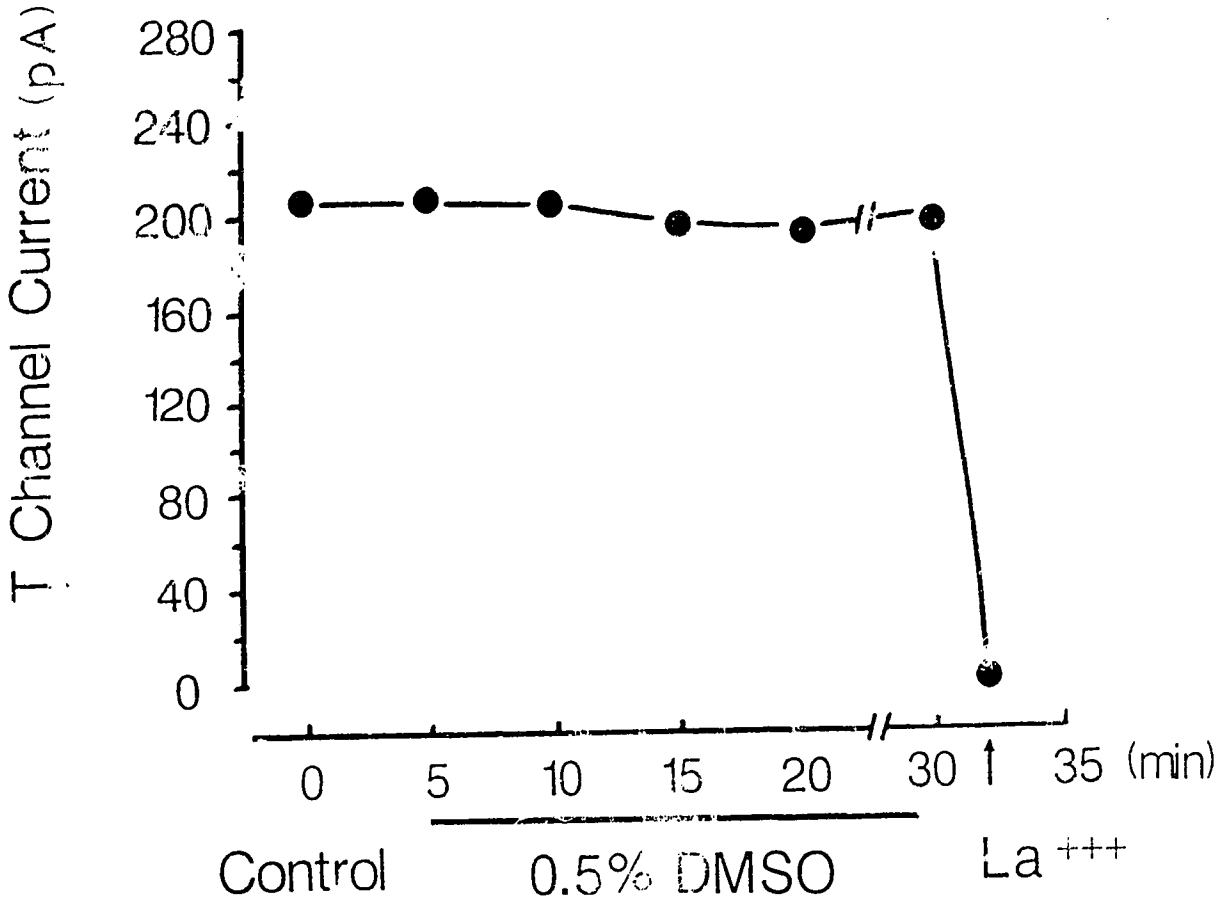

T Channel Current (pA)
280
240
200
160
120
80
40
0
0
5
10
15
20
30
35 (min)
Control
0.5% DMSO
La +++

Fig.IV-8. The effect of 0.5% DMSO on the T channel current in one N1E-115 cell. Peak T channel currents were elicited at the membrane potential of -20 mV from a holding potential of -80 mV. The peak T channel current was obtained in the absence, and then presence of DMSO (0.5%, v/v) followed by the application of 2 mM La^{+++}. DMSO alone (0.5%) had no effect on the amplitude of T channel currents.

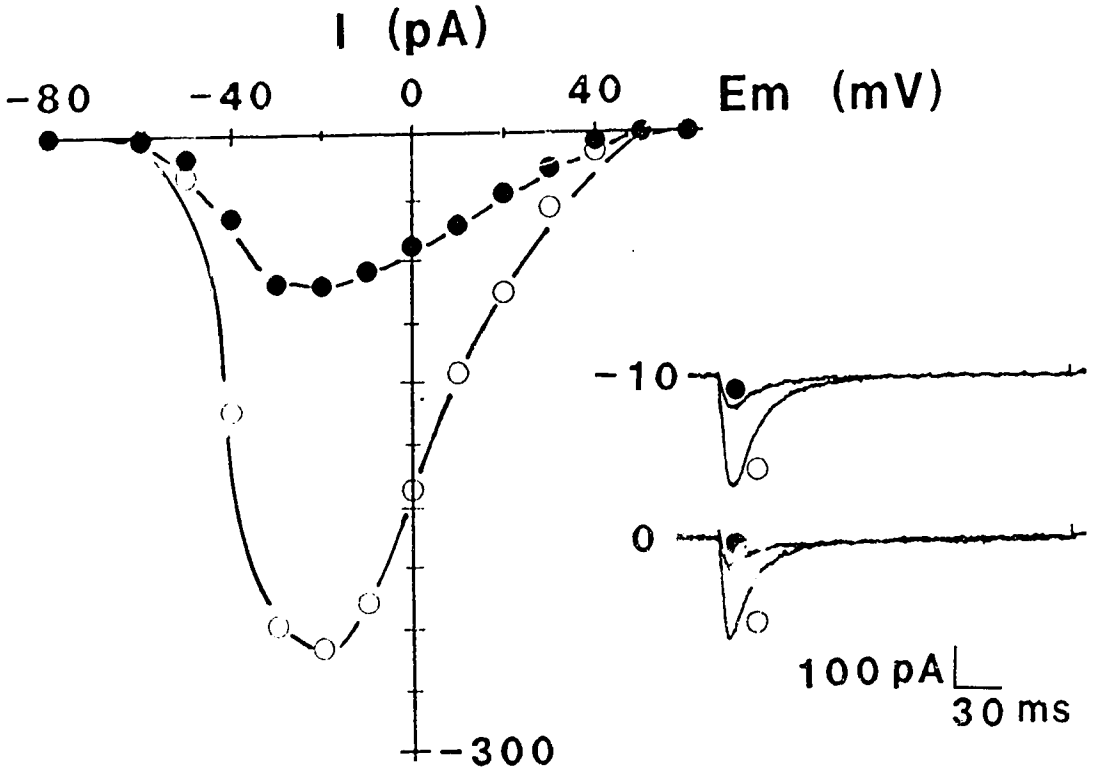

I (pA)
-80
-40
0
40
Em (mV)
-300
-10
0
100 pA
30 ms

Fig.IV-9. The effect of nifedipine/DMSO on T channel currents after pretreatment of the cell with acetone. One N1E-115 cell was pretreated with 0.3% acetone for 10 min (open circles) before nifedipine/DMSO (30 μM) was applied to the same cell (filled circles). The original T channel current traces at two depolarization levels (mV) are shown. Holding potential was -80 mV. Leakage and capacitive currents were subtracted from the original current traces. Pretreatment of the cell with acetone did not modify the effect of nifedipine/DMSO on T channel currents.

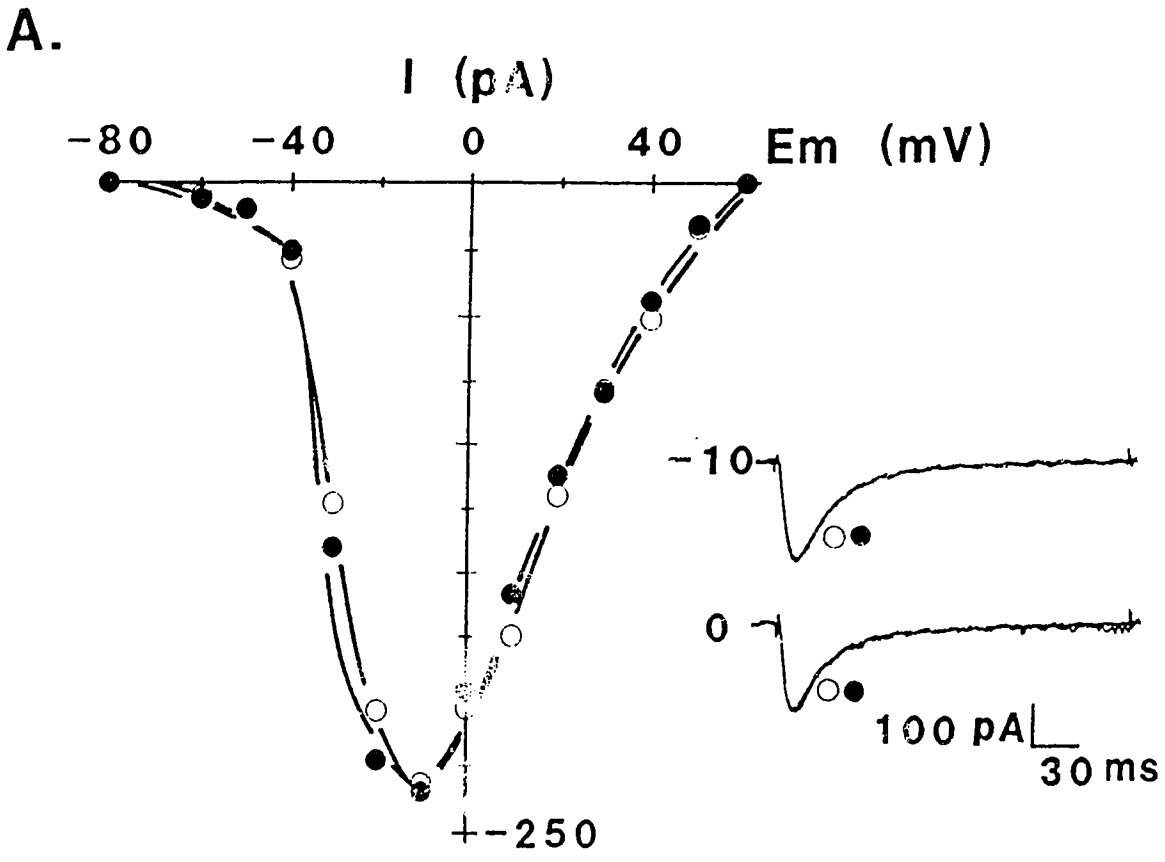

A.
I (pA)
-80
-40
0
40
Em (mV)
-10
0
100 pA
30 ms
-250

Fig.IV-10. The effect of nifedipine/acetone on T channel currents after pretreatment of the cell with DMSO. Open circles represent the T channel currents recorded after 10 min pretreatment of the cell with 0.3% DMSO. Filled circles represent the T channel currents recorded after nifedipine/acetone (30 μM) was applied to the same cell. Also shown are the original T channel current traces at two test potential levels (mV). Holding potential was set at -80 mV. Leakage and current were subtracted from the original current traces. Pretreatment of did not render the T channel sensitive to nifedipine/acetone.

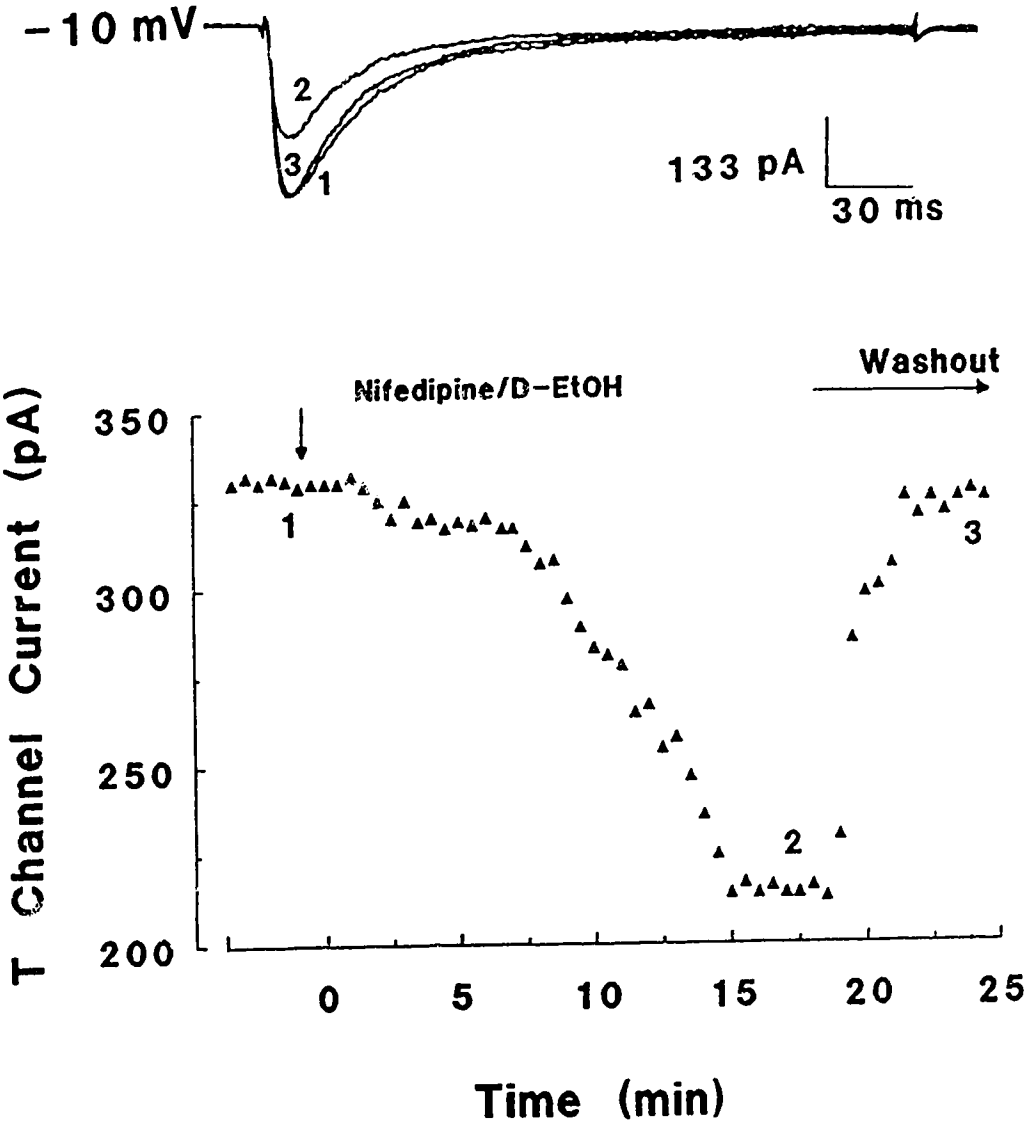

-10 mV
2
3
1
133 pA
30 ms
Nifedipine/D-EtOH
Washout
T Channel Current (pA)
350
300
250
200
1
2
3
0
5
10
15
20
25
Time (min)

Fig.IV-11. The inhibition of T channel currents by dri·l nifedipine/DMSO powder redissolved in ethanol (nifedipine/D-EtOH) in one N1E-115 cell. Peak T channel currents were elicited at the membrane potential of -10 mV from holding potential of -80 mV. The peak current was obtained in the absence, and then presence of 10 μM nifedipine/D-EtOH followed by "washout" with drug-free bath solution. The suppression of the T channel current reached a maximum at the 15th min after the application of the agent. Representative T channel current traces are shown on at the top of the figure.

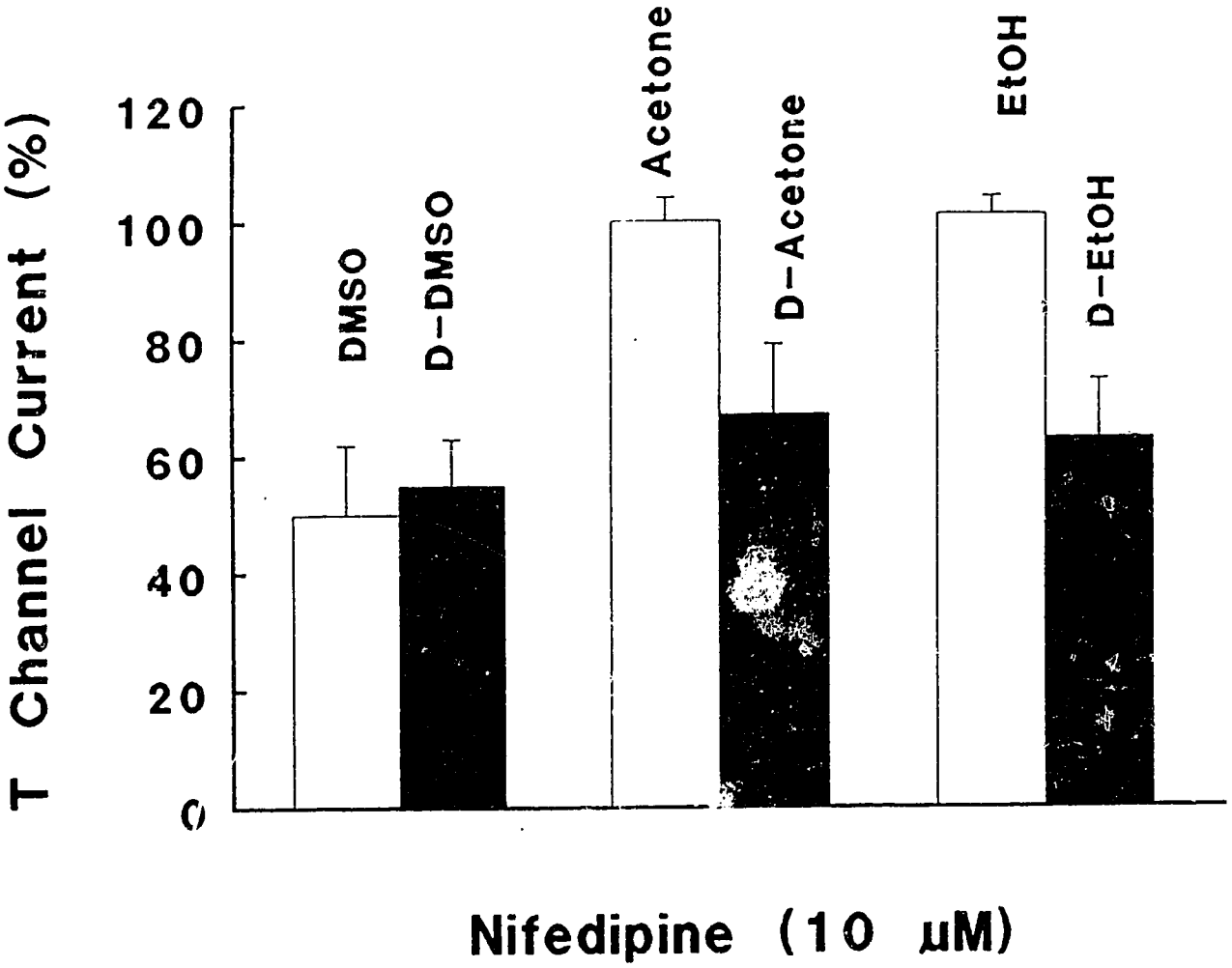
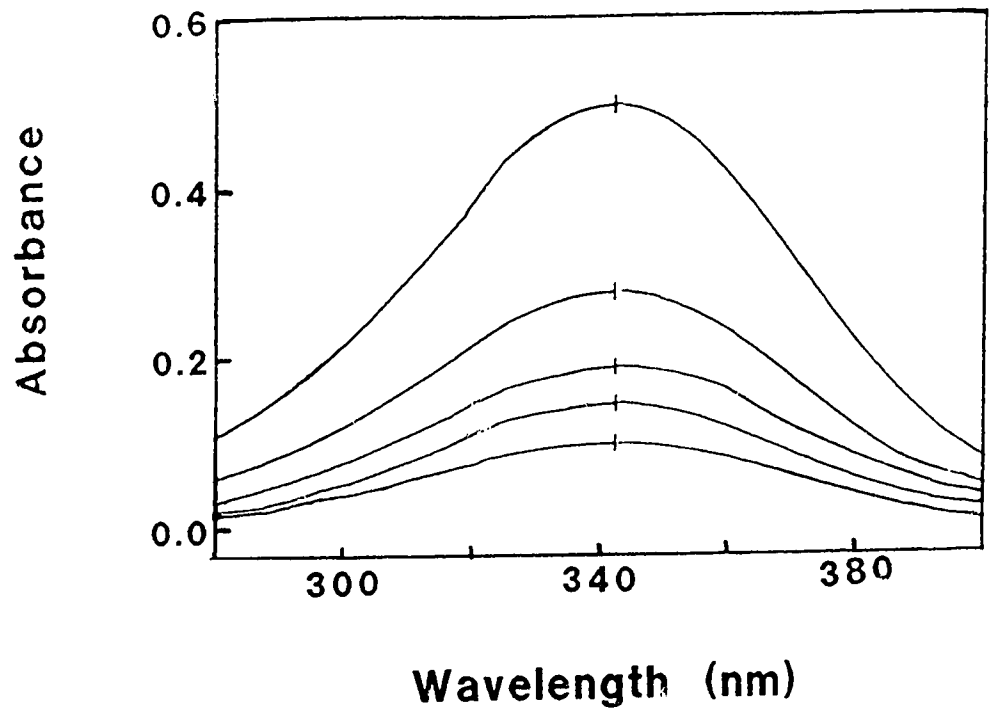

T Channel Current (%)
120
100
80
60
40
20
0
DMSO
D-DMSO
Acetone
D-Acetone
EtOH
D-EtOH
Nifedipine (10 μM)

Fig.IV-12. Effects of different nifedipine preparations on T channel currents in N1E-115 cells. The solvents used are indicated above each bar. The prefix "D-" indicates that nifedipine was dissolved in DMSO, dried, and then redissolved in various solvents. Each preparation of nifedipine was tested in 5 or more cells. Bars represent the SEM.

A.


0.6
0.4
0.2
0.0
Absorbance
300
340
380
Wavelength (nm)

B.

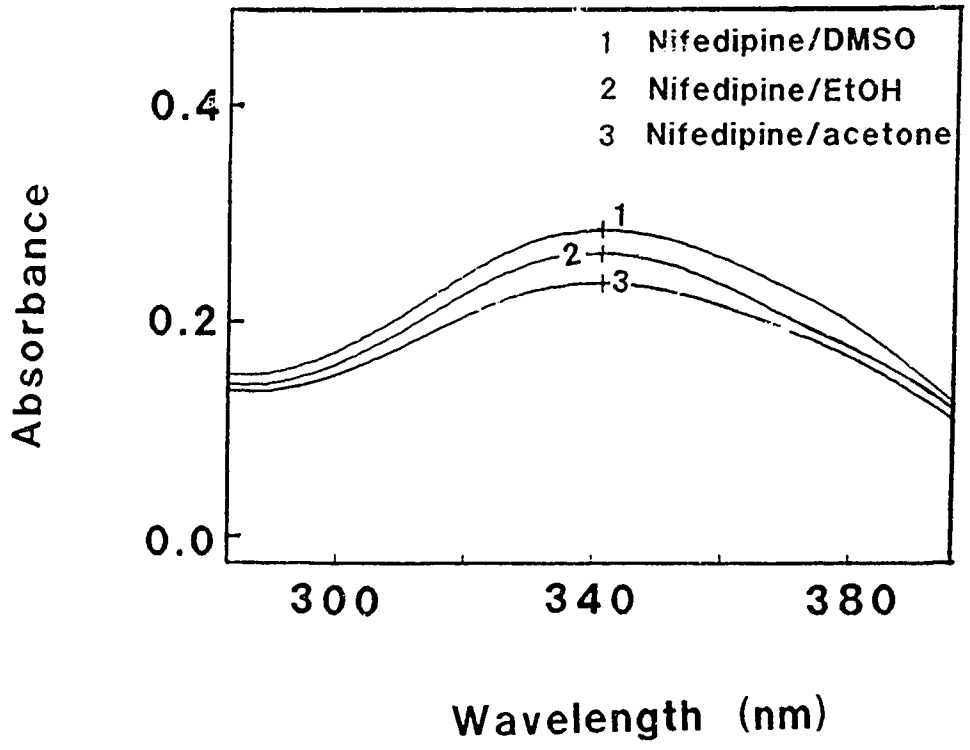

1 Nifedipine/DMSO
2 Nifedipine/EtOH
3 Nifedipine/acetone
0.4
0.2
0.0
Absorbance
1
2
3
300
340
380
Wavelength (nm)

Fig.IV-13. Absorption spectra of nifedipine dissolved in DMSO, acetone or ethanol at different concentrations. **A.** Absorption spectra of nifedipine/DMSO at concentrations of 10, 20, 30, 50, 100 μM (bottom to top). **B.** Absorption spectra of nifedipine/DMSO, nifedipine/acetone, and nifedipine/EtOH at a concentration of 50 μM. The absorbance maximum of these three preparations were at the same wavelength (342 nm).

B.

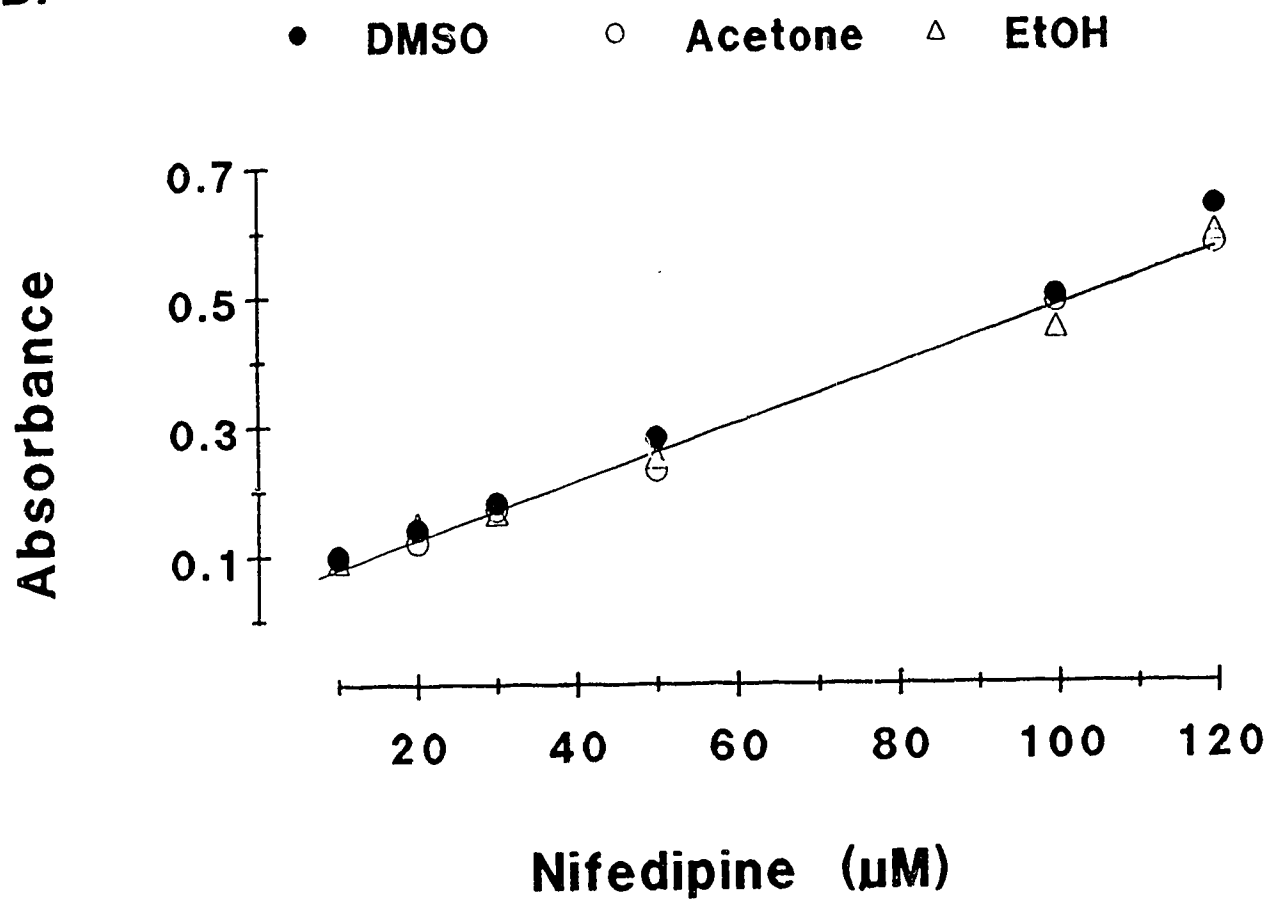

DMSO
Acetone
EtOH
Absorbance
0.7
0.5
0.3
0.1
20
40
60
80
100
120
Nifedipine (µM)

Fig.IV-14. The relationship between the absorbance maximum (342 nm) and concentrations of nifedipine/DMSO, nifedipine/acetone, or nifedipine/EtOH. The points were fitted by least square regression. The linear plot indicated that precipitation of nifedipine did not occur within the concentration range tested.

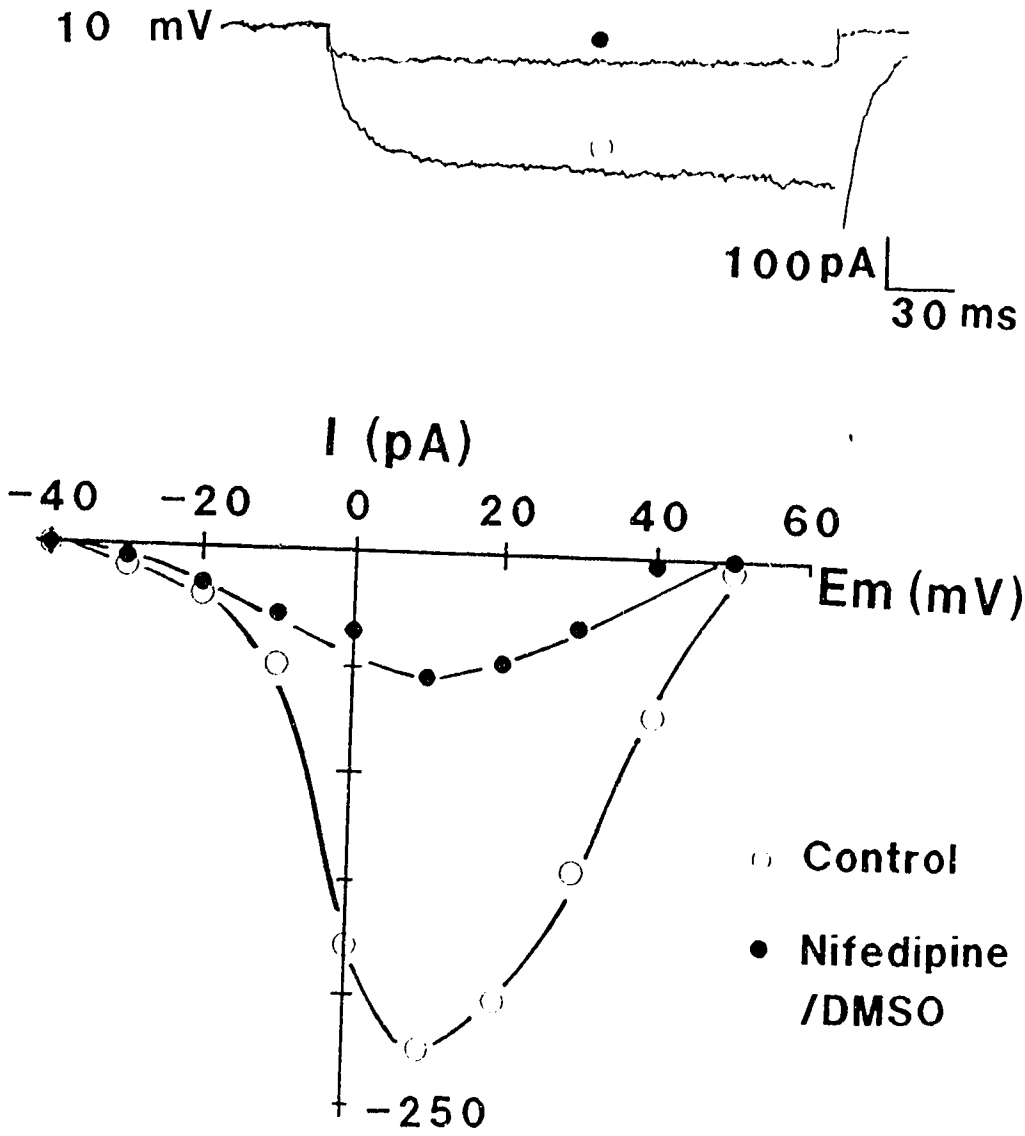

10 mV
100pA
30 ms
I (pA)
-40
-20
0
20
40
60
Em (mV)
-250
Control
Nifedipine
/DMSO

Fig.IV-15. Effect of nifedipine/DMSO on L channel currents in one N1E-115 cell. L channel currents were recorded in the absence (open circles) and then presence (filled circles) of 1 μM nifedipine/DMSO. The L channel current was activated at -30 mV from a holding potential of -40 mV. The peak of the I-V relationship of L channels (10 mV) and the apparent reversal potential (50 mV) were not changed by nifedipine/DMSO. Leakage and capacitive currents were subtracted from the original traces.

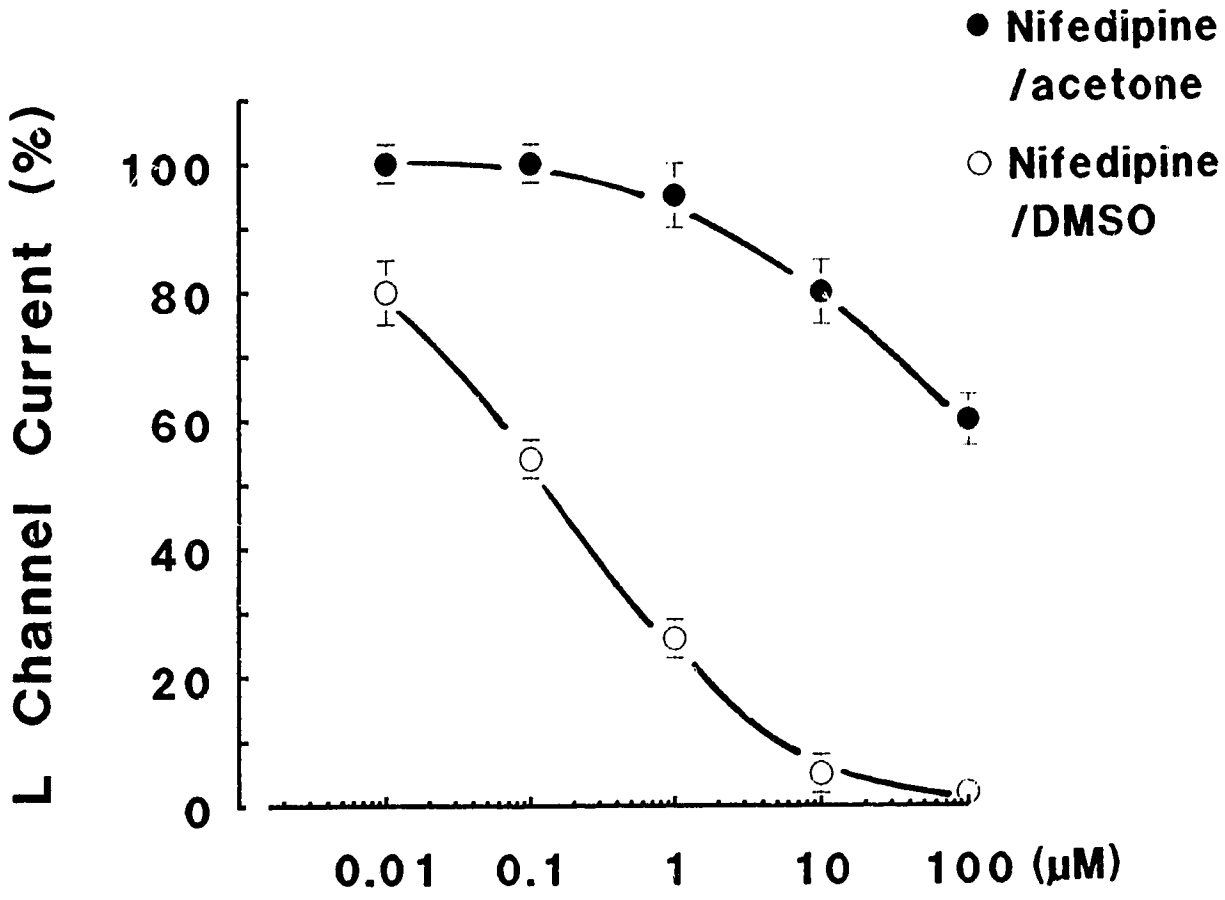

● Nifedipine /acetone
○ Nifedipine /DMSO
L Channel Current (%)
100
80
60
40
20
0
0.01
0.1
1
10
100 (μM)

Fig.IV-16. The dose-response of the L channel current to nifedipine dissolved in DMSO or acetone. Four to seven cells were examined at each dose of nifedipine/DMSO or nifedipine/acetone. The amplitude of L channel currents in the absence of nifedipine preparations was taken as the control (100%). The final concentration of DMSO or acetone in the bath was fixed at 0.3% for each dose of nifedipine.

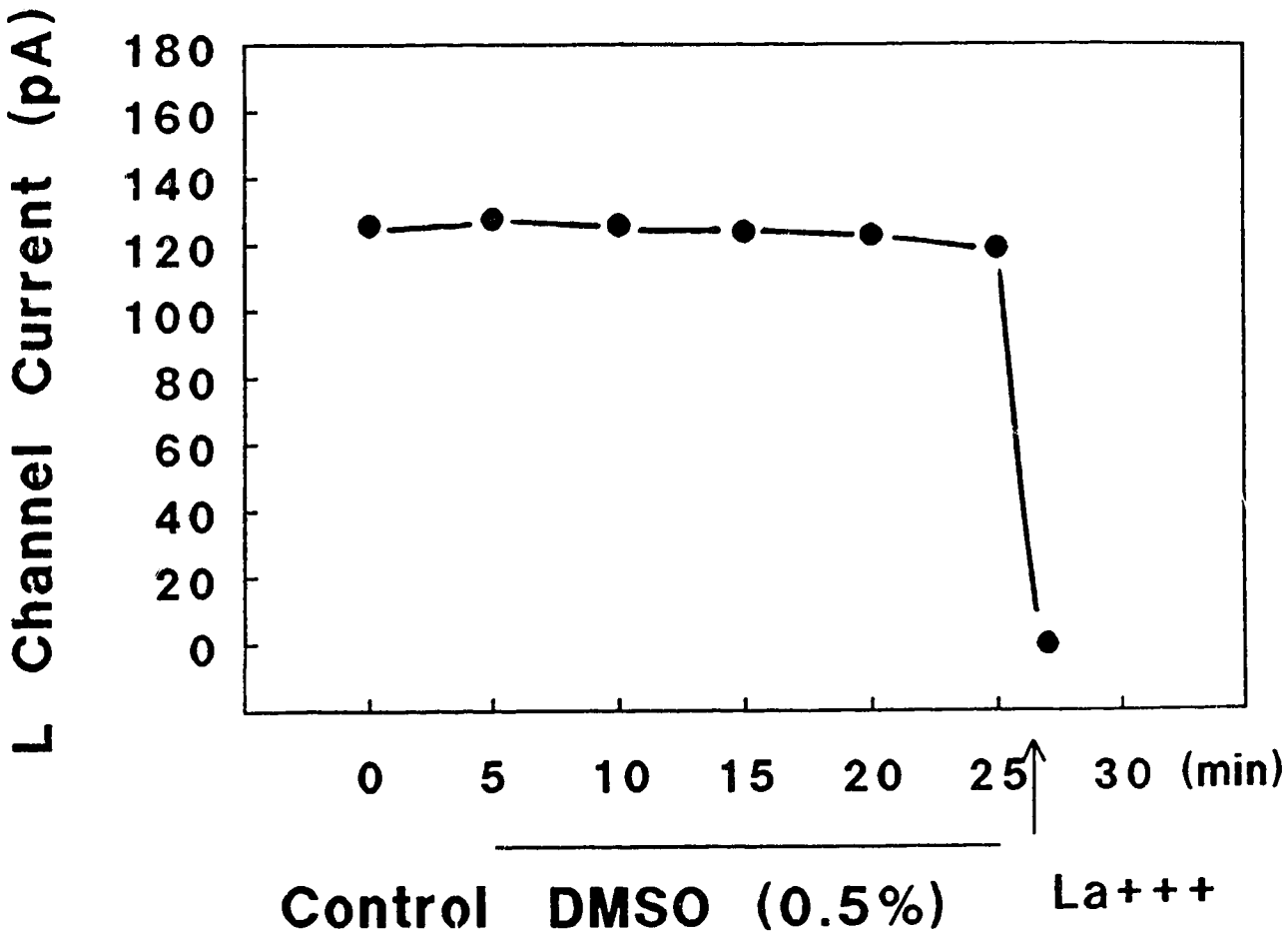

L Channel Current (pA)
180
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30 (min)
Control
DMSO (0.5%)
La+++

Fig.IV-17. The effect of 0.5% DMSO on the L channel current in one N1E-115 cell. The peak L channel current was elicited at the membrane potential of 10 mV from a holding potential of -40 mV. There was no change in the amplitude of L channel currents in the presence of 0.5% DMSO for 25 min. The subsequent application of 2 mM La^{+++} completely abolished L channel currents.

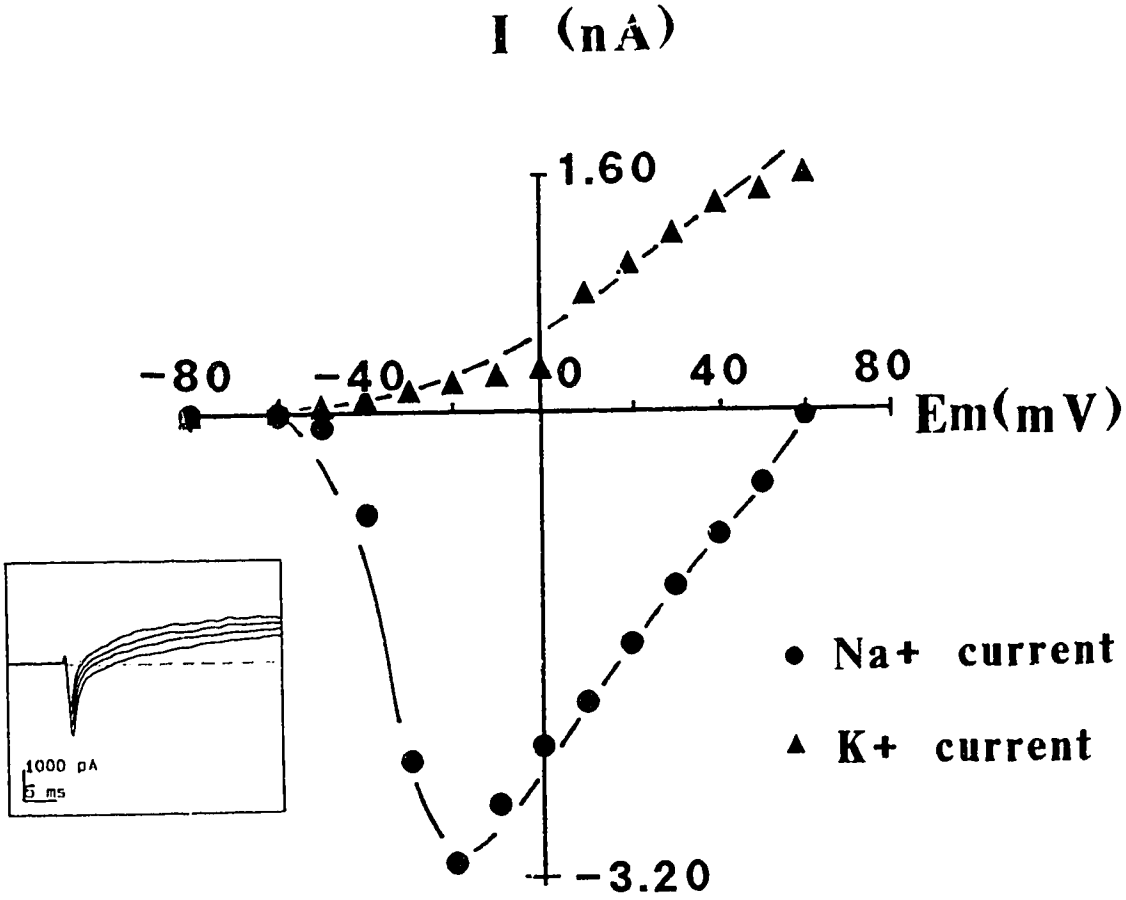

I (nA)
1.60
-80
-40
0
40
80
Em(mV)
-3.20
Na+ current
K+ current
1000 pA
5 ms

Fig. IV-18. The Na^+ and K^+ channel current records and associated current-voltage relationship in one N1E-115 cell. Holding potential was set at -80 mV. Leakage and capacitive currents were subtracted. The composition of the pipette and the bath solutions was chosen to record Na^+ and K^+ channel currents (see Chapter 2 for details). The Na^+ channel current was activated at -50 mV, peaked at -20 mV, and reversed at about 60 mV. The K^+ channel current was activated at -60 mV. The inset shows the representative Na^+ and K^+ channel current traces elicited from different test pulses (10 to 40 mV). The time course of Na^+ channel currents was characterized by fast activation (1-1.5 msec) and fast inactivation (half time, 2-3 msec). The outward K^+ channel currents were not inactivated during the 30 msec period of the depolarizing pulse.

CHAPTER 5

The Effects of Bay K-8644 on Calcium Channel Currents

5.1. The effect of Bay K-8644 dissolved in DMSO (Bay K-8644/DMSO) on the T channel current.

The effect of Bay K-8644/DMSO on the T channel current in one N1E-115 cell is shown in Fig.V-1. Bay K-8644/DMSO at a concentration of 0.6 μM inhibited T channel currents in this cell by 45%. The I-V relationship of T channels did not shift in the presence of Bay K-8644/DMSO (Fig.V-1B). The potential at which T channel currents were activated and the apparent reversal potential were also not changed. After the effect of Bay K-8644/DMSO on T channel currents was established, the bath solution containing Bay K-8644/DMSO was replaced at an exchange rate of 1.4 ml/10 sec to wash out Bay K-8644/DMSO from the bath. Five min after the initiation of wash-out, the T channel currents almost completely recovered, indicating that the inhibitory effect of Bay K-8644/DMSO on the T channel current was reversible. The reversibility of the inhibitory effect of Bay K-8644/DMSO eliminates time dependent deterioration or run down of Ca^{++} channels as an explanation for the decrease in T channel current.

With a fixed DMSO concentration in the bath (0.3%), Bay K-8644/DMSO inhibited T channel currents and this inhibition was concentration dependent (Fig.V-2). A 37% inhibition of T channel currents by Bay K-8644/DMSO was detected at a concentration of 0.1 μM ($n=6$, $p<0.05$). At a concentration of 10 μM, Bay K-8644/DMSO inhibited the T channel current by 89% ($n=8$, $p<0.05$).

The effect of Bay K-8644/DMSO on the steady-state inactivation of T channel currents was investigated in five N1E-115 cells. Although the absolute amplitudes of T channel currents at various conditioning potentials were decreased by Bay K-8644/DMSO (Fig.V-3A), the normalized steady-state inactivation curve was not significantly shifted by 0.6 μM Bay K-8644/DMSO (Fig.V-3B).

5.2. The effect of Bay K-8644 dissolved in ethanol (Bay K-8644/EtOH) or Bay K-8644 dissolved in PEG (Bay K-8644/PEG) on the T channel current.

Bay K-8644/EtOH or Bay K-8644/PEG at the concentration range from 0.1 to 100 μM had no effect on the amplitude, kinetics or I-V relationship of T channel currents. Fig. V-4 and Fig. V-5 show the representative T channel current records which were the same before or after the application of 10 μM Bay K-8644/EtOH and 10 μM Bay K-8644/PEG, respectively.

5.3. The effect of solvents on the T channel current.

The amplitude, kinetics and I-V relationship of T channel currents in N1E-115 cells were not changed in the presence of 0.3% or 0.5% DMSO, 0.3% EtOH or 0.3%

PEG (see Chapter 4, section 4.4.).

Whether the presence of some solvents in the bath changes the T channel sensitivity to Bay K-8644 was studied in the following experiments. N1E-115 cells were pretreated with 0.3% EtOH for 10 min and then Bay K-8644/DMSO was added to the bath. In the presence of EtOH, an obvious inhibition of the T channel current by 1 μM Bay K-8644/DMSO was still observed (Fig.V-6). There was no significant difference in the inhibition of T channel currents by 1 μM Bay K-8644/DMSO with or without pretreatment of the cells with 0.3% EtOH ($p > 0.05$). When DMSO was first added to the bath at a final concentration of 0.3% for 10 min, subsequent addition of Bay K-8644/EtOH (10 μM) to the same bath still had no effect on T channel currents (Fig.V-7). The data show that the interaction of solvents with T channels is not the determining factor in the effect of Bay K-8644/EtOH or Bay K-8644/DMSO on T channel currents.

5.4. The Interaction of Bay K-8644/DMSO with Bay K-8644/EtOH or Bay K-8644/PEG.

In order to examine whether Bay K-8644/DMSO and Bay K-8644/EtOH or Bay K-8644/PEG act on the same site(s) on or near T-type calcium channels, the inhibitory effect of Bay K-8644/DMSO on the T channel currents in the presence of Bay K-8644/EtOH was examined in six cells. A concentration of 10 μM Bay K-8644/EtOH was first obtained in the bath solution. No change in either amplitude or kinetics of T channel currents could be detected. Five minutes later, Bay K-

8644/DMSO (0.1 μM) was added to the bath. The T channel current was reduced to 57% of the control level (Fig.V-4). The inhibition of the T channel current (Fig.V-5) was also observed when Bay K-8644/DMSO (1 μM) was added after the treatment of cells with 10 μM Bay K-8644/PEG. This group of experiments consisted of 6 cells. The inhibitory effect of Bay K-8644/DMSO on the T channel current was not significantly different ($p > 0.05$) when cells pretreated with Bay K-8644/EtOH or Bay K-8644/PEG were compared.

5.5. The effect of dried Bay K-8644/DMSO, redissolved in EtOH or PEG, on the T channel current.

The inhibitory effect of Bay K-8644/DMSO on T channel currents may depend on a specific effect of DMSO which may cause a conformational change in Bay K-8644. Evidence supporting this hypothesis is shown by the next group of experiments. Bay K-8644 was first dissolved in DMSO at a concentration of 30 mM and, then, dried using a flash evaporator at 50°C for 24 hr in the dark. The dried Bay K-8644/DMSO powder was then re-dissolved in DMSO, EtOH, or PEG. Bay K-8644/DMSO re-dissolved in DMSO (Bay K-8644/D-DMSO) decreased T channel currents by 45 $\pm$ 5% at a concentration of 0.6 μM in four cells (Fig.V-8). There was no significant difference in the inhibitory effect or the onset of the inhibition of T channel currents induced by either 0.6 μM Bay K-8644/DMSO or Bay K-8644/D-DMSO ($p > 0.05$). When dried Bay K-8644/DMSO powder was re-dissolved in EtOH (Bay K-8644/D-EtOH) or PEG (Bay K-8644/D-PEG), redissolved Bay K-8644

preparations inhibited T channel currents. At a concentration of 0.6 μM, Bay K-8644/D-EtOH decreased the T channel current by 39 $\pm$ 5% (n=5, $p<0.05$), while Bay K-8644/D-PEG inhibited the T channel current by 35 $\pm$ 5% (n=5, $P<0.05$) (Fig.V-8). The inhibitory effect of Bay K-8644/DMSO redissolved in DMSO, EtOH or PEG on T channel currents could be reversed by washout procedures. Further examination of the onset time for the effect of Bay K-8644 on T channel currents revealed that the maximum effect of Bay K-8644/DMSO and Bay K-8644/D-DMSO was established 5 min after the addition of the agent, whereas the maximum effect of Bay K-8644/D-EtOH or Bay K-8644/D-PEG on T channel currents required more time (> 7 min). These results suggest that the molecular conformation of Bay K-8644 may be modified by DMSO. Hence, when Bay K-8644 was subsequently dissolved in EtOH or PEG, it became capable of inhibiting T channel currents in N1E-115 cells. The delay in the onset of the action of Bay K-8644/DMSO re-dissolved in either EtOH or PEG suggests that these two solvents may also exert some effects on Bay K-8644.

5.6. The solubility of Bay K-8644 dissolved in different solvents.

When the stock solutions of Bay K-8644 are added to the bath, it is possible that some degree of precipitation of Bay K-8644 from different solvent preparations may occur. If this were the case, the effect of Bay K-8644/DMSO on T channel currents could be due to less precipitation of Bay K-8644 with DMSO as the solvent. The present results, however, were not consistent with this hypothesis. Bay K-8644

at stock solution concentrations of 0.3 μM to 3 mM was completely dissolved in DMSO, EtOH, or PEG. These Bay K-8644 stock solutions were added to the bath solution to reach different final concentrations. The concentration of solvents in the bath was always kept at 0.3%. Under these conditions, absorbance measurement showed that the absorbance maximum of Bay K-8644/DMSO at different concentrations (10 to 100 μM) in the bath varied, but always occurred at the same wavelength, i.e. 422 nm (Fig.V-9). The absorbance maximum of Bay K-8644/EtOH or Bay K-8644/PEG at different concentrations also occurred at 422 nm (Fig.V-9). Fig.V-10 shows that the absorbance maximum of Bay K-8644 dissolved in DMSO, EtOH, or PEG rose as the concentration of Bay K-8644 in the bath was increased. There was no significant difference in the absorbance maximum of Bay K-8644 dissolved in DMSO, EtOH, or PEG at each of the different concentrations. The linear plot of absorbance maximum vs. Bay K-8644 concentrations (Fig.V-10) indicated that precipitation of Bay K-8644 in the bath solution within the concentration range used in this study was unlikely.

5.7. The effect of Bay K-8644 dissolved in different solvents on the L channel current.

Dissolved in DMSO or in EtOH, Bay K-8644 increased the amplitude of L channel currents as expected (Karpinski *et al.*, 1989). However, the effects of Bay K-8644/DMSO and Bay K-8644/EtOH on the L channel currents were different. Bay K-8644/DMSO (100 nM) increased L channel currents by 139% (Fig.V-11). At

the same concentration, however, Bay K-8644/EtOH only increased L channel currents by 60%, as shown in Fig.V-12.

Fig.V-13 shows the dose-response of the L channel current to Bay K-8644 dissolved in DMSO or EtOH. The effects of Bay K-8644/EtOH are approximately one order of magnitude less effective than Bay K-8644/DMSO within the concentration range examined. At a concentration of 100 nM (n=4), Bay K-8644/EtOH was only about 66% as effective as Bay K-8644/DMSO (n=5).

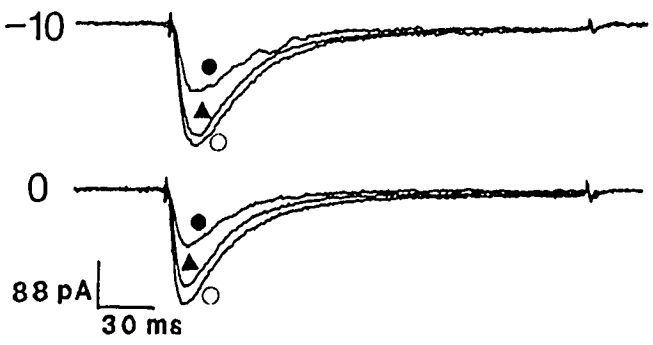
5.8. The effect of solvents on the L channel current

The amplitude, I-V relationship, and kinetics of the L channel current were not affected by DMSO or EtOH at the concentration used in the present studies (see Chapter 4, section 4.9.)

5.9. The effect of Bay K-8644/DMSO on Na^+ and K^+ channel currents.

The specificity of the Bay K-8644/DMSO effect on calcium channel currents was further investigated by examining its effect on Na^+ and K^+ channel currents in N1E-115 cells. Bay K-8644/DMSO (10 μM) failed to modulate either Na^+ or K^+ channel currents in four N1E-115 cells. One example is shown in Fig.V-14.

A.


-10
0
88 pA
30 ms

B.

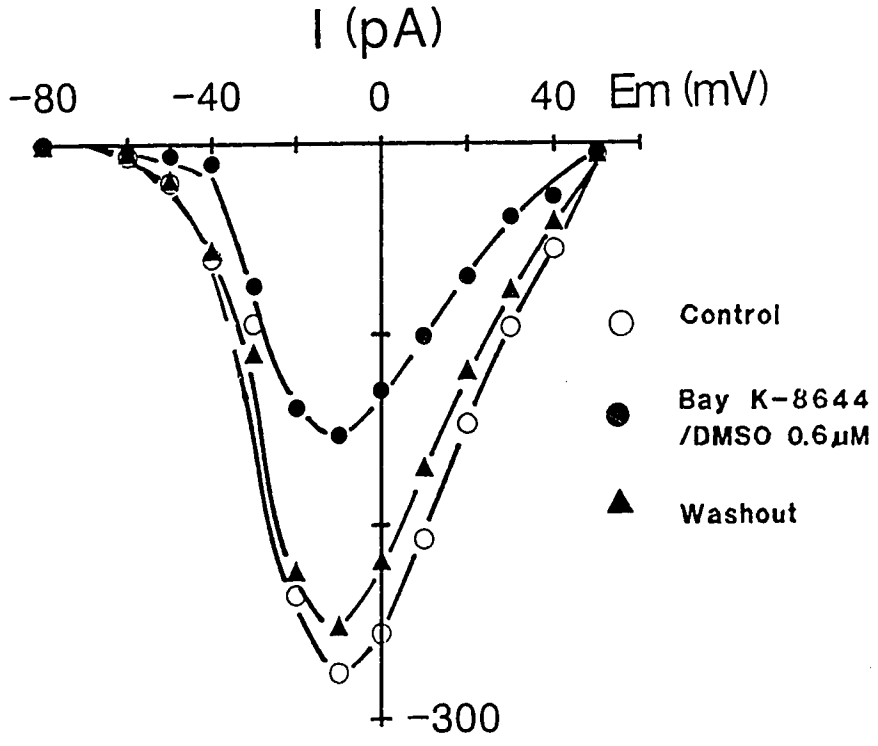

I (pA)
-80
-40
0
40
Em (mV)
-300
Control
Bay K-8644
/DMSO 0.6μM
Washout

Fig.V-1. The inhibition of T channel currents by Bay K-8644/DMSO in one N1E-115 cell. **A.** Original traces of T channel currents were obtained in the absence (open circles) and then presence of 0.6 μM Bay K-8644/DMSO (filled circles) followed by a 5 min washout (filled triangles) in the same cell. After washout the T channel current recovered to 91% of the control level (open circles). Test pulses are indicated in mV beside each trace. Leakage and capacitive currents were subtracted. **B.** I-V relationships of the T channel were obtained under different conditions as described above. Holding potential was set at -80 mV. The peak of the I-V relationship of T channels (-10 mV) and the apparent reversal potential (+50 mV) were not affected by Bay K-8644/DMSO.

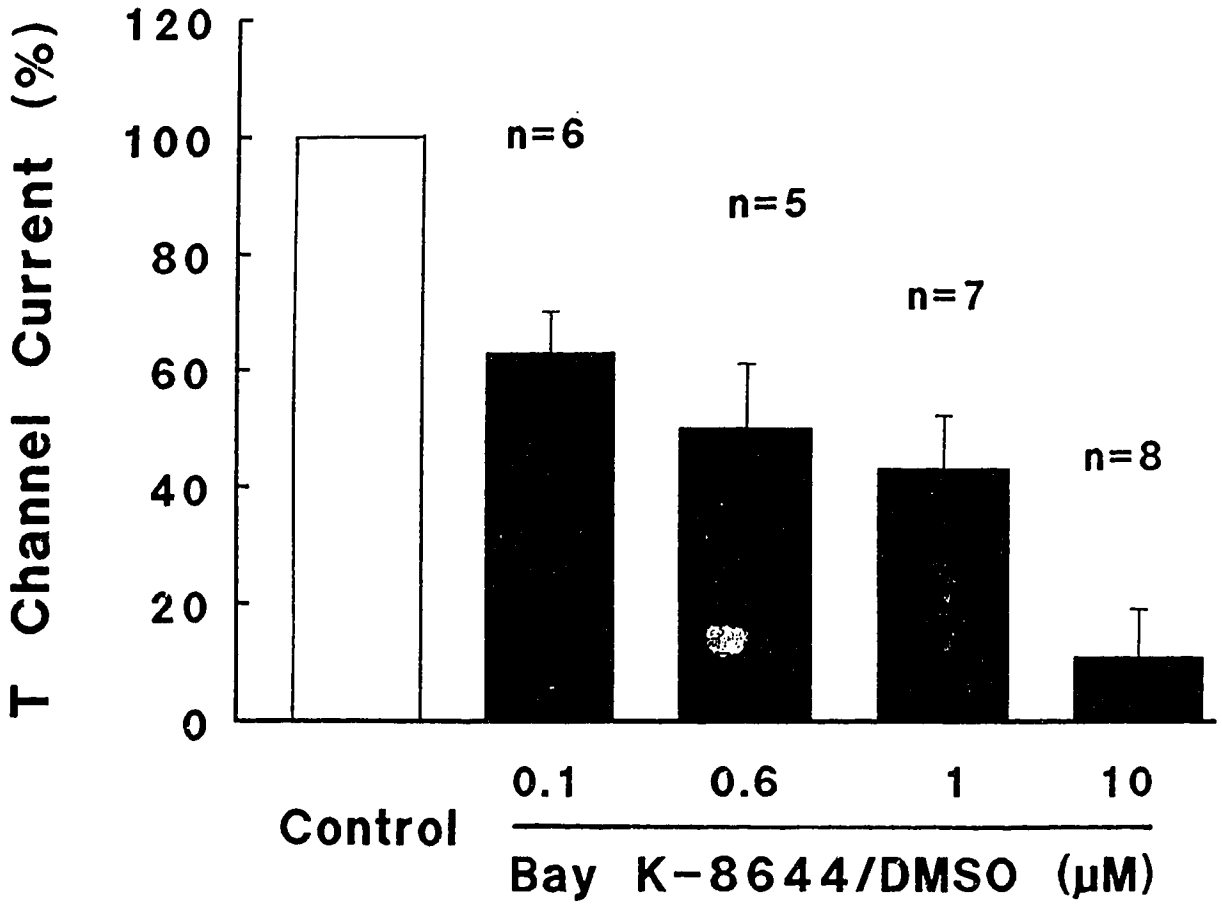

T Channel Current (%)
120
100
80
60
40
20
0
n=6
n=5
n=7
n=8
Control
0.1
0.6
1
10
Bay K-8644/DMSO (μM)

Fig.V-2. Dose-response of T channel currents to Bay K-8644/DMSO. The inhibition of T channel currents by Bay K-8644/DMSO was expressed as the percentage of the control (100%). 100 nM Bay K-8644/DMSO inhibited the T channel current by 37% ($p<0.05$). 10 μM Bay K-8644/DMSO reduced the amplitude of the T channel current to 11% of the control level ($p<0.05$). Bars represent the SEM.

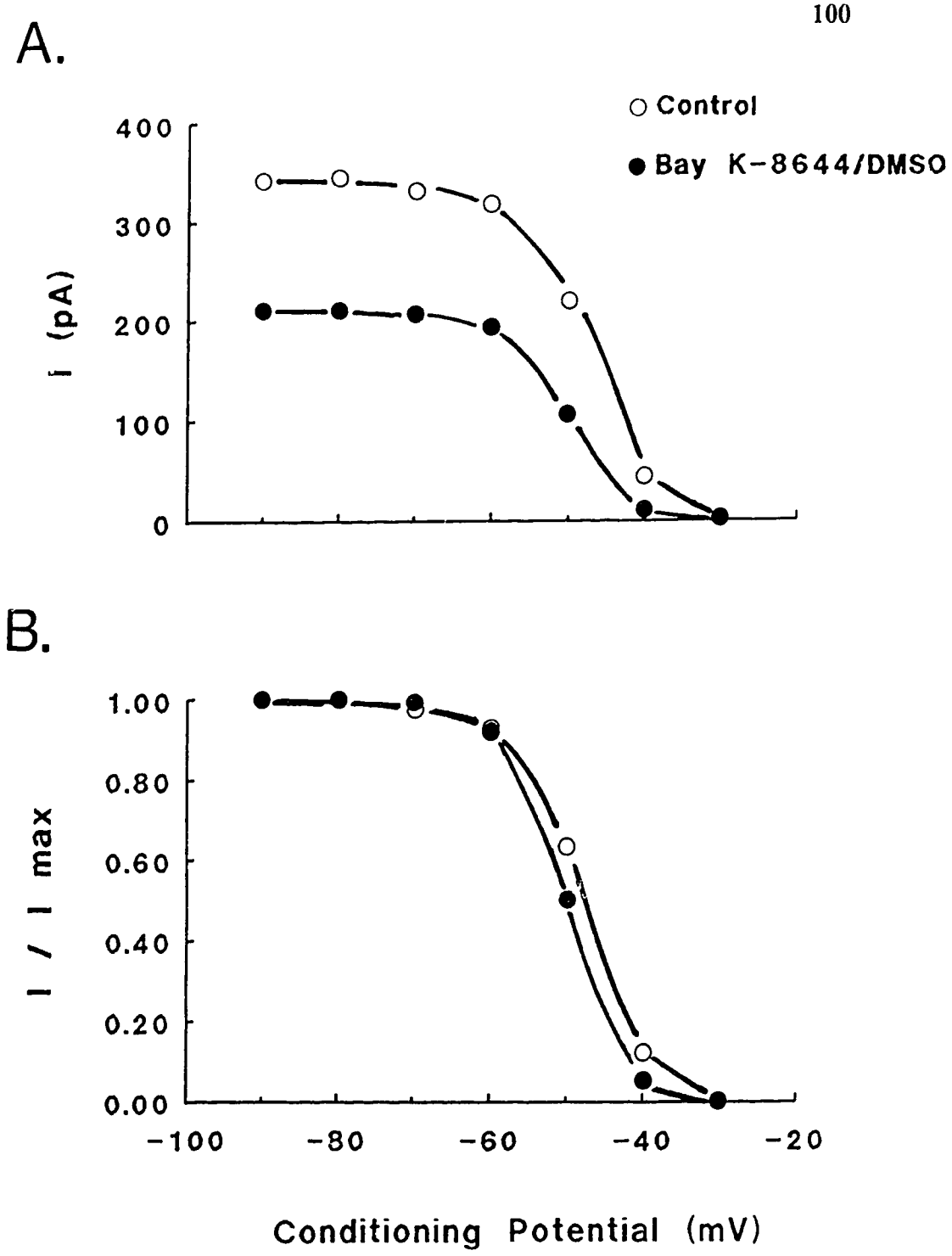

A.
Control
Bay K-8644/DMSO
400
300
200
100
0
i (pA)
B.
1.00
0.80
0.60
0.40
0.20
0.00
I / I max
-100
-80
-60
-40
-20
Conditioning Potential (mV)

Fig.V-3. The steady-state inactivation of T channel currents in response to 0.6 μM Bay K-8644/DMSO in one N1E-115 cell. The conditioning potential was changed in a stepwise manner with a fixed membrane depolarization to -20 mV. The membrane potential was held for 2 sec at each conditioning potential. **A.** The absolute steady-state inactivation curve for T channels was significantly suppressed by Bay K-8644/DMSO. **B.** The normalized steady-state inactivation curves for T channels in the absence or presence of Bay K-8644/DMSO were not different. The smooth curves in **A** and **B** were fitted by eye.

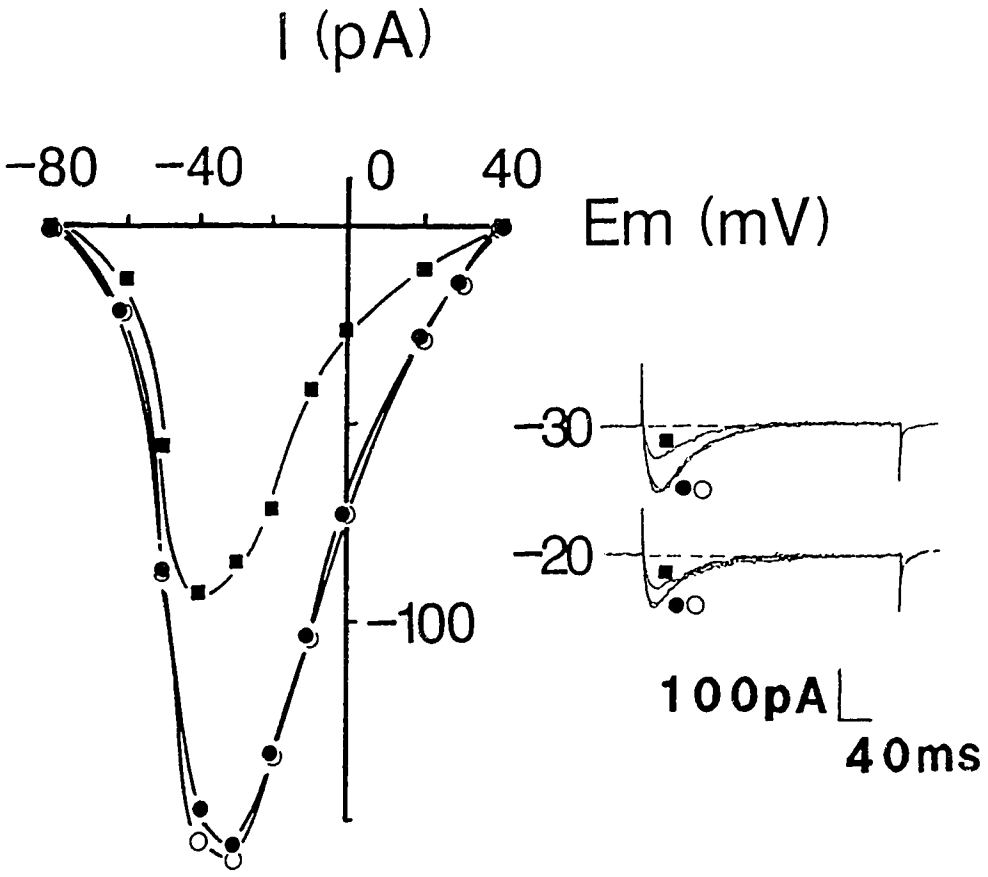
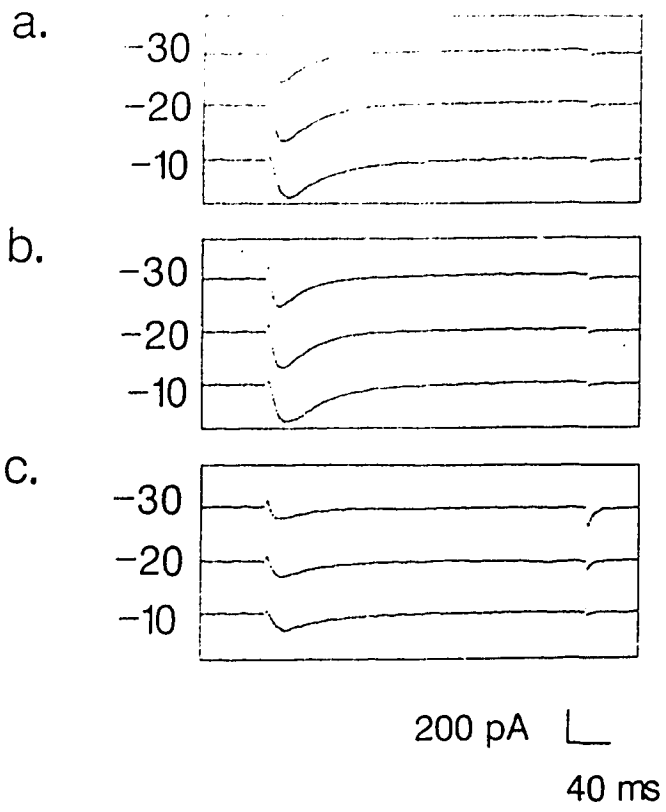

I (pA)
−80
−40
0
40
Em (mV)
−100
−30
−20
100pA
40ms

Fig.V-4. The effects of Bay K-8644/DMSO on T channel currents after pretreatment of the cell with Bay K-8644/EtOH. Control currents were first recorded in the absence of any agents (open circles). Then, Bay K-8644/EtOH was added to the bath to reach a final concentration of 10 μM (filled circles). Subsequently, Bay K-8644/DMSO (100 nM) was applied to the same cell (filled squares). Leakage and capacitive currents were subtracted from the original current traces. Holding potential was set at -80 mV. Representative original T channel current traces are also shown at two depolarization levels (mV).

A.



B.

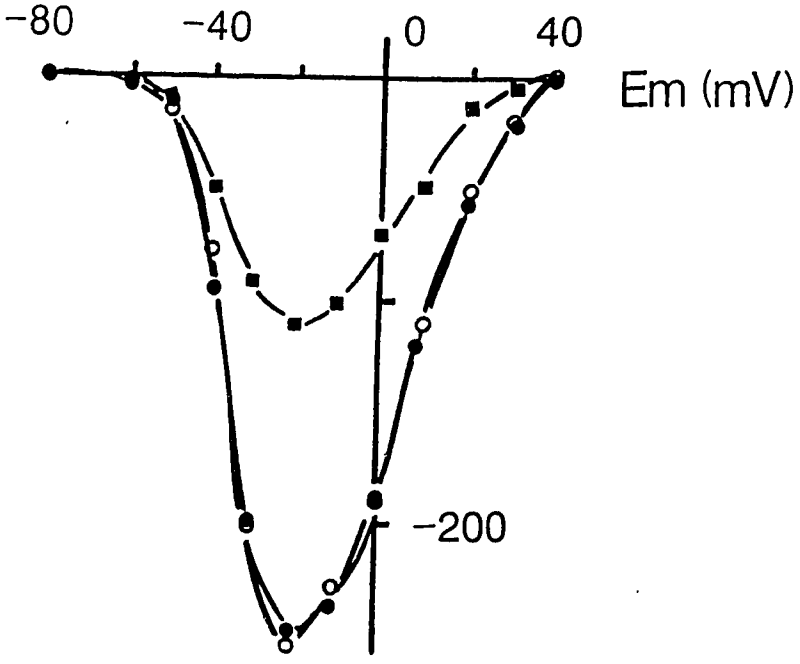


Fig. V-5. The effect of Bay K-8644/DMSO on T channel currents after pretreatment of the cell with Bay K-8644/PEG. **A.** The original traces of T channel currents were recorded prior to addition of any drugs (shown in a). Bay K-8644/PEG (10 μM) was then applied (shown in b) and followed by 1 μM Bay K-8644/DMSO to the same cell (shown in c). Test pulses are indicated in mV beside each trace. Leakage and capacitive currents were subtracted. **B.** After the control recording of the I-V relationship was made (open circles), Bay K-8644/PEG (filled circles) was added to the bath solution to reach a final concentration of 10 μM, and was followed by Bay K-8644/DMSO at a final concentration of 1 μM (filled squares). Bay K-8644/PEG had no effect on the T channel current, but Bay K-8644/DMSO produced a significant decrease in T channel currents.

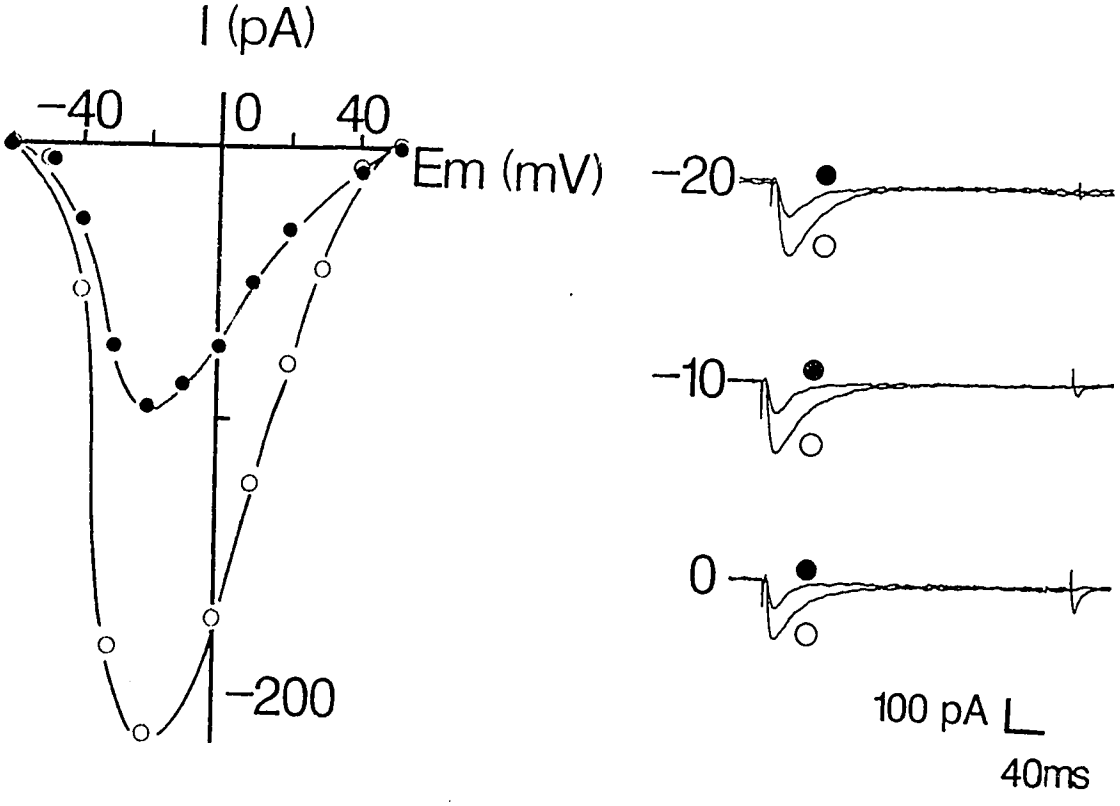

I (pA)
−40
0
40
Em (mV)
−200
−20
−10
0
100 pA
40ms

Fig.V-6. The effect of Bay K-8644/DMSO on T channel currents after pretreatment of the cell with EtOH. One cell was pretreated with 0.3% EtOH for 10 min (open circles) before 1 μM Bay K-8644/DMSO was applied to the cell (filled circles). The I-V relationships and the original current traces of T channels are shown in the left and right panels, respectively. Bay K-8644/DMSO inhibited the T channel current in the presence of 0.3% EtOH.

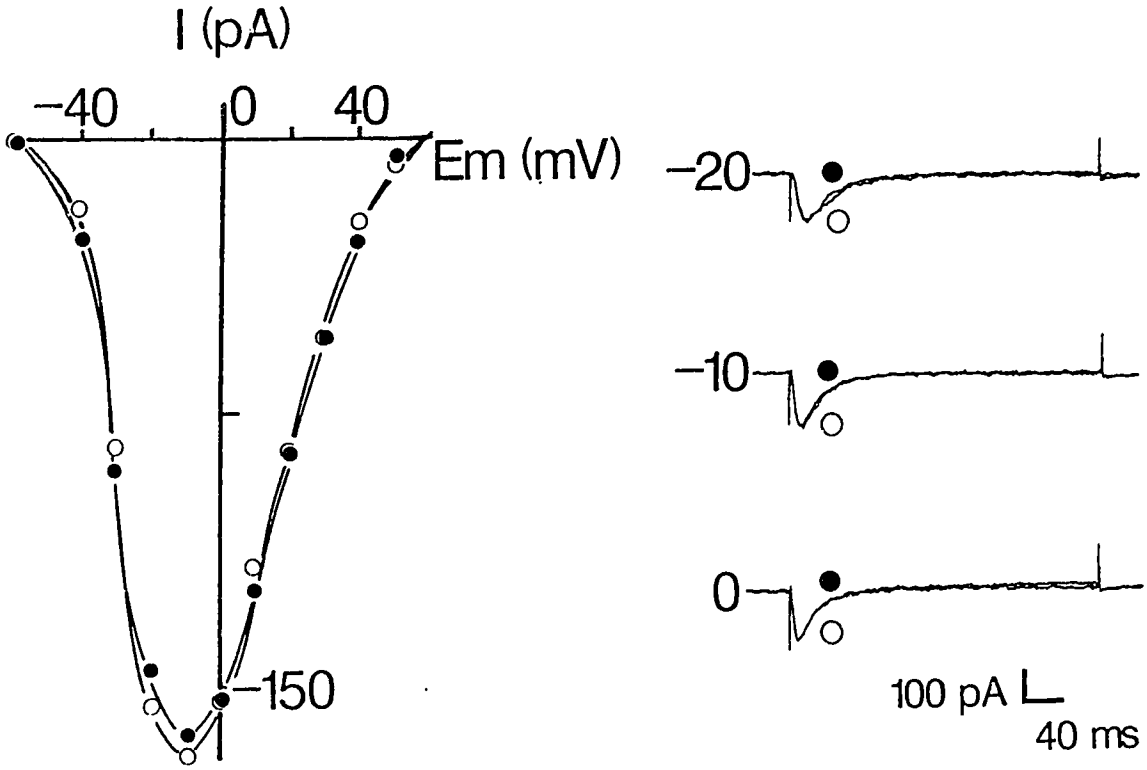

I (pA)
−40
0
40
Em (mV)
−150
−20
−10
0
100 pA
40 ms

Fig.V-7. The effect of Bay K-8644/EtOH on T channel currents after pretreatment of the cell with DMSO. Open circles represent T channel currents recorded in the presence of DMSO (0.3%). Filled circles represent T channel currents recorded after 1 μM Bay K-8644/EtOH was applied to the same cell. The I-V relationships and the original current traces of T channels are shown in the left and right panels, respectively. Bay K-8644/EtOH had no effect on the T channel current when the cell was pretreated with 0.3% DMSO for 10 min.

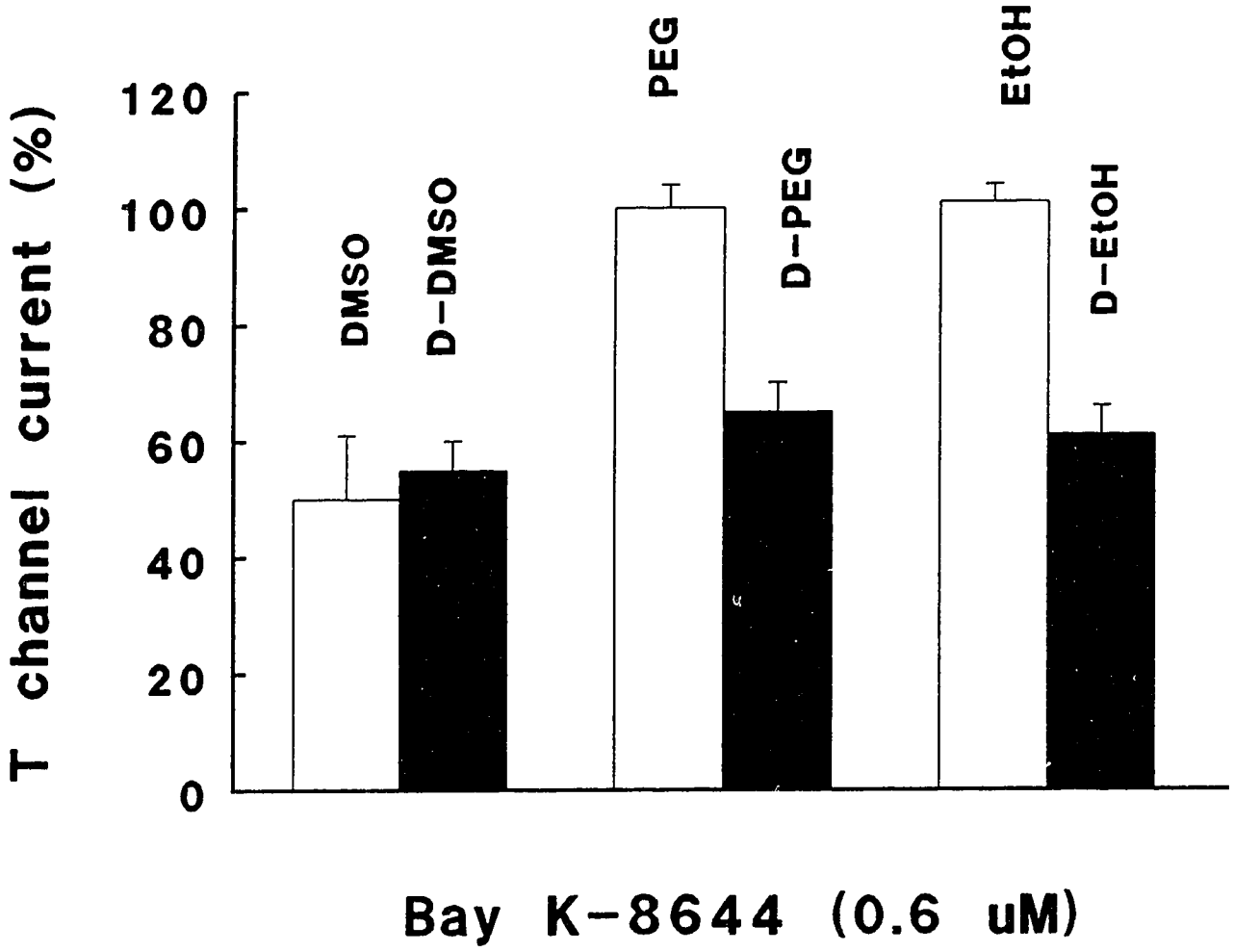
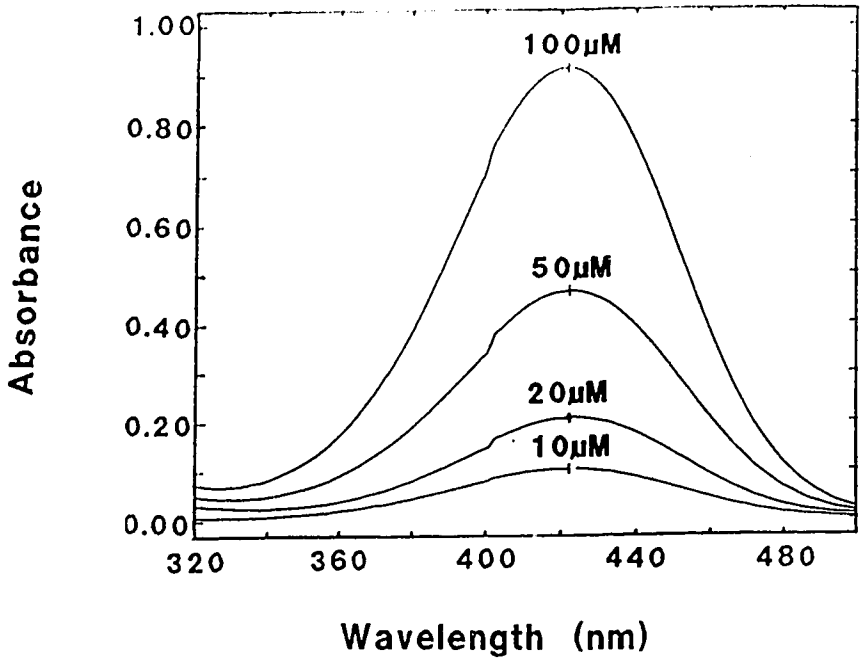

T channel current (%)
120
100
80
60
40
20
0
DMSO
D-DMSO
PEG
D-PEG
EtOH
D-EtOH
Bay K-8644 (0.6 uM)

Fig.V-8. Effects of redissolved Bay K-8644/DMSO on T channel currents. 0.6 μM Bay K-8644/DMSO decreased T channel currents by 50%. At the same concentration, Bay K-8644/PEG and Bay K-8644/EtOH had no effects on T channel currents. Bay K-8644/D-DMSO (0.6 μM) inhibited the T channel current by 55% ($n=4$, $p<0.05$). The amplitude of T channel currents was reduced to 65% and 61% of the control level by Bay K-8644/D-PEG ($n=5$, $p<0.05$) and Bay K-8644/D-EtOH ($n=5$, $p<0.05$) at the same concentration of 0.6 μM, respectively. The solvents used to dissolve or re-dissolve Bay K-8644 are indicated above each bar. Bars represent the SEM.

A.


1.00
0.80
0.60
0.40
0.20
0.00
Absorbance
100μM
50μM
20μM
10μM
320
360
400
440
480
Wavelength (nm)

B.

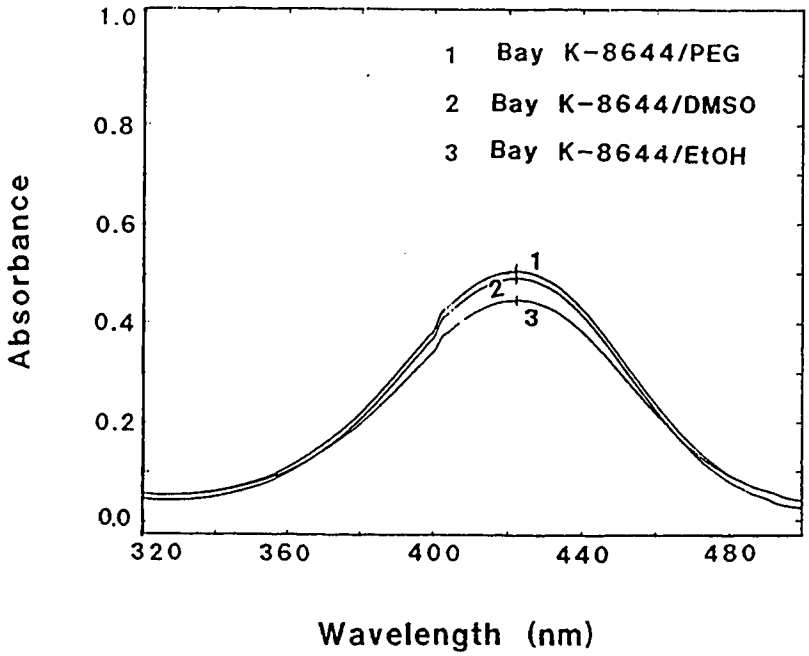

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
1 Bay K-8644/PEG
2 Bay K-8644/DMSO
3 Bay K-8644/EtOH
1
2
3
320
360
400
440
480
Wavelength (nm)

Fig.V-9. Absorption spectra of Bay K-8644 dissolved in DMSO, PEG, or EtOH at different concentrations. **A.** Absorption spectra of Bay K-8644/DMSO at concentrations of 10, 20, 50, and 100 μM (bottom to top). The absorbance maximum of Bay K-8644/DMSO at various concentrations was detected at a wavelength of 422 nm. **B.** Absorption spectra of Bay K-8644/DMSO, Bay K-8644/PEG, and Bay K-8644/EtOH at the concentration of 50 μM. The absorbance maximums of these three preparations occurred at the same wavelength (422 nm).

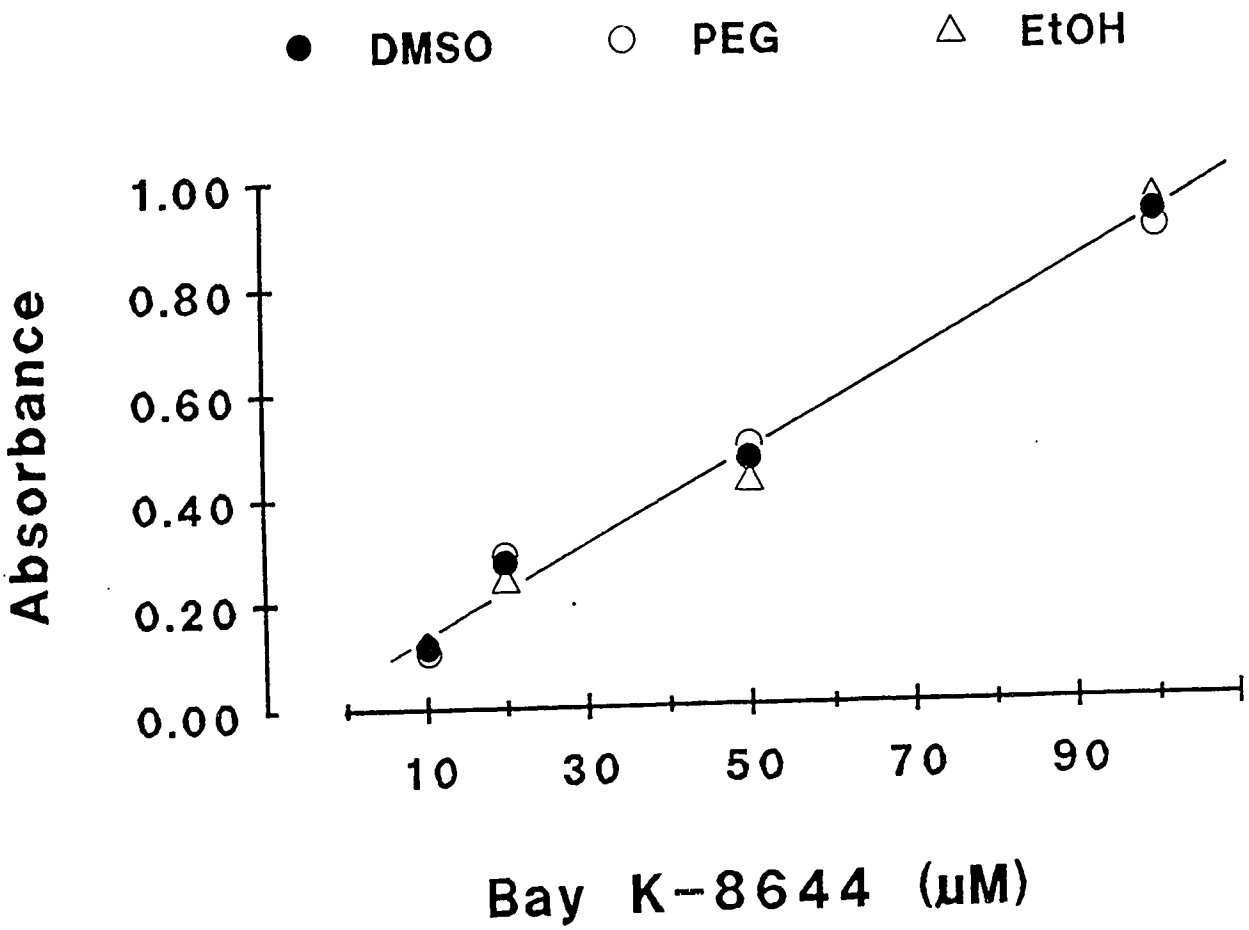
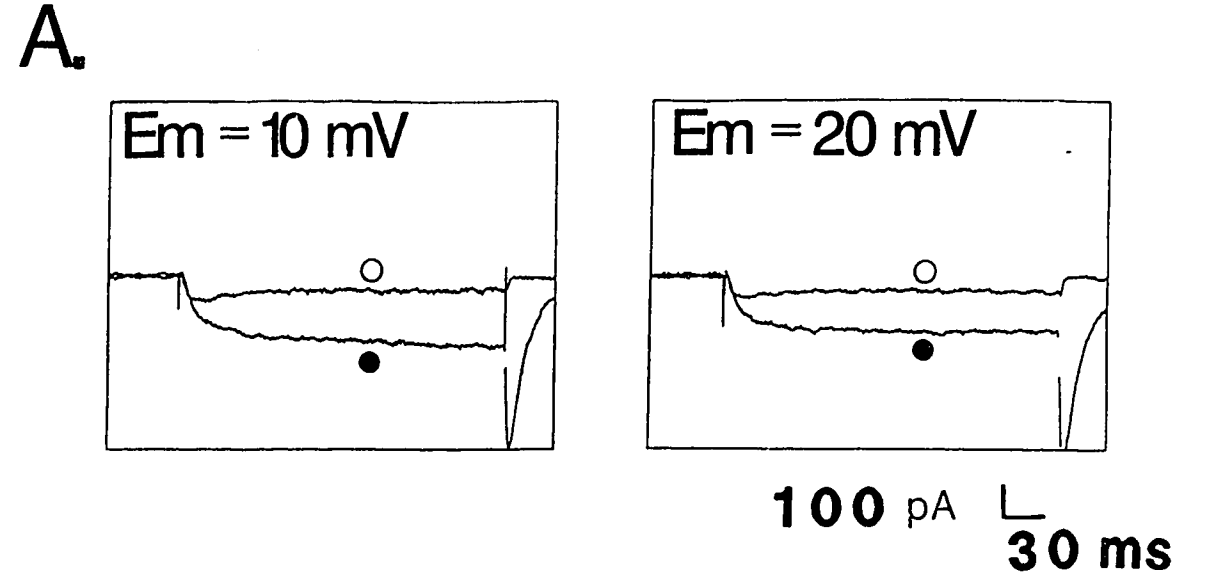

● DMSO
○ PEG
△ EtOH
Absorbance
1.00
0.80
0.60
0.40
0.20
0.00
10
30
50
70
90
Bay K-8644 (μM)

Fig.V-10. The relationship between the absorbance maximum and concentrations of Bay K-8644/DMSO, Bay K-8644/PEG, and Bay K-8644/EtOH. The points were fitted by least square regression. The linear relations indicated that no precipitation of Bay K-8644 occurred within the concentration range tested.


A.
Em = 10 mV
Em = 20 mV
100 pA
30 ms

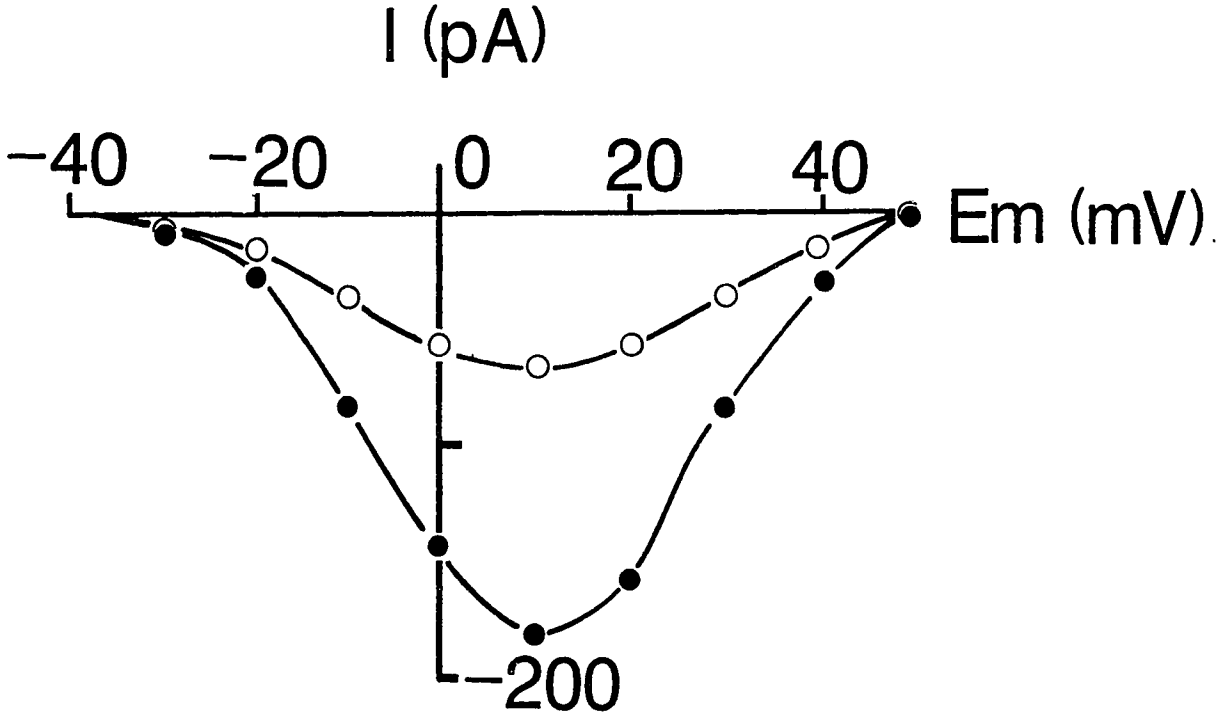
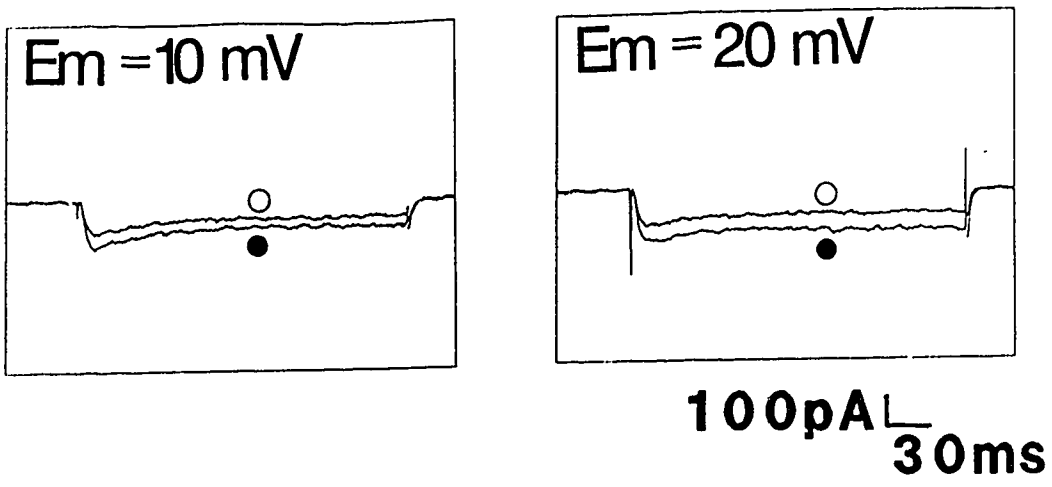

I (pA)
−40
−20
0
20
40
Em (mV)
−200

Fig.V-11. Effects of Bay K-8644/DMSO on L channel currents in one N1E-115 cell. Holding potential was set at -40 mV. Leakage and capacitive currents were subtracted. The records with open circles or filled circles were obtained before and after application of 100 nM Bay K-8644/DMSO, respectively. Bay K-8644/DMSO increased the amplitude of L channel currents.

B.



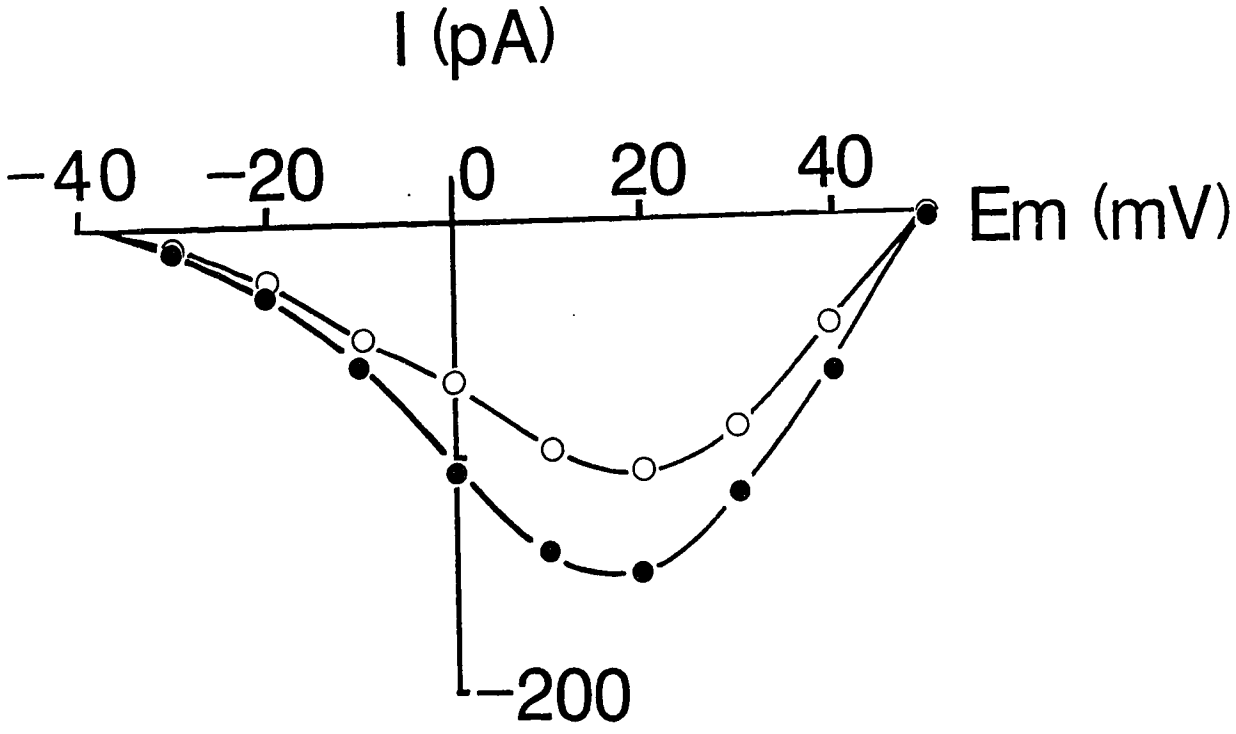


Fig.V-12. The effect of Bay K-8644/EtOH on L channel currents in one N1E-115 cell. The curves were obtained in the absence (open circles) and presence (filled circles) of 100 nM Bay K-8644/EtOH, respectively. Bay K-8644/EtOH increased the amplitude of L channel currents. The leakage and capacitive currents were subtracted from original current traces. Holding potential was -40 mV.

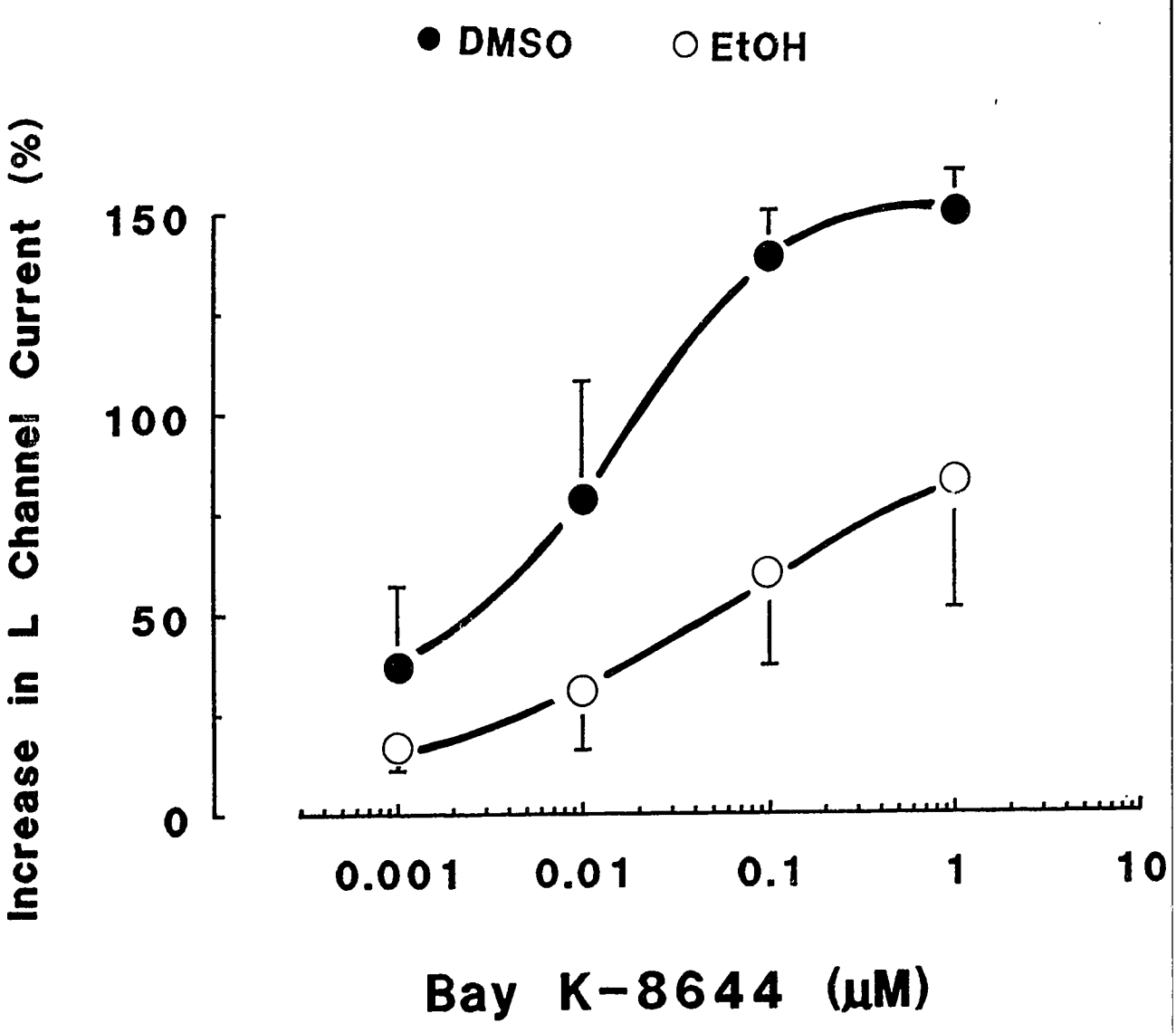

● DMSO
○ EtOH
Increase in L Channel Current (%)
150
100
50
0
0.001
0.01
0.1
1
10
Bay K-8644 (μM)

Fig.V-13. Dose-dependent effects of Bay K-8644/DMSO (filled circles) and Bay K-8644/EtOH (open circles) on L channel currents in N1E-115 cells. Data are expressed as mean ± SEM and 4 to 6 cells were examined at each concentration for Bay K-8644/DMSO or Bay K-8644/EtOH. Bay K-8644/DMSO (1 nM) increased the peak L channel currents by 38%. At a concentration of 1 μM, Bay K-8644/DMSO maximally increased the L channel current by 149%. Bay K-8644/DMSO was a more effective L channel agonist than was Bay K-8644/EtOH within the concentration range tested. The final concentration of DMSO and EtOH in the bath solution was fixed at 0.3%.

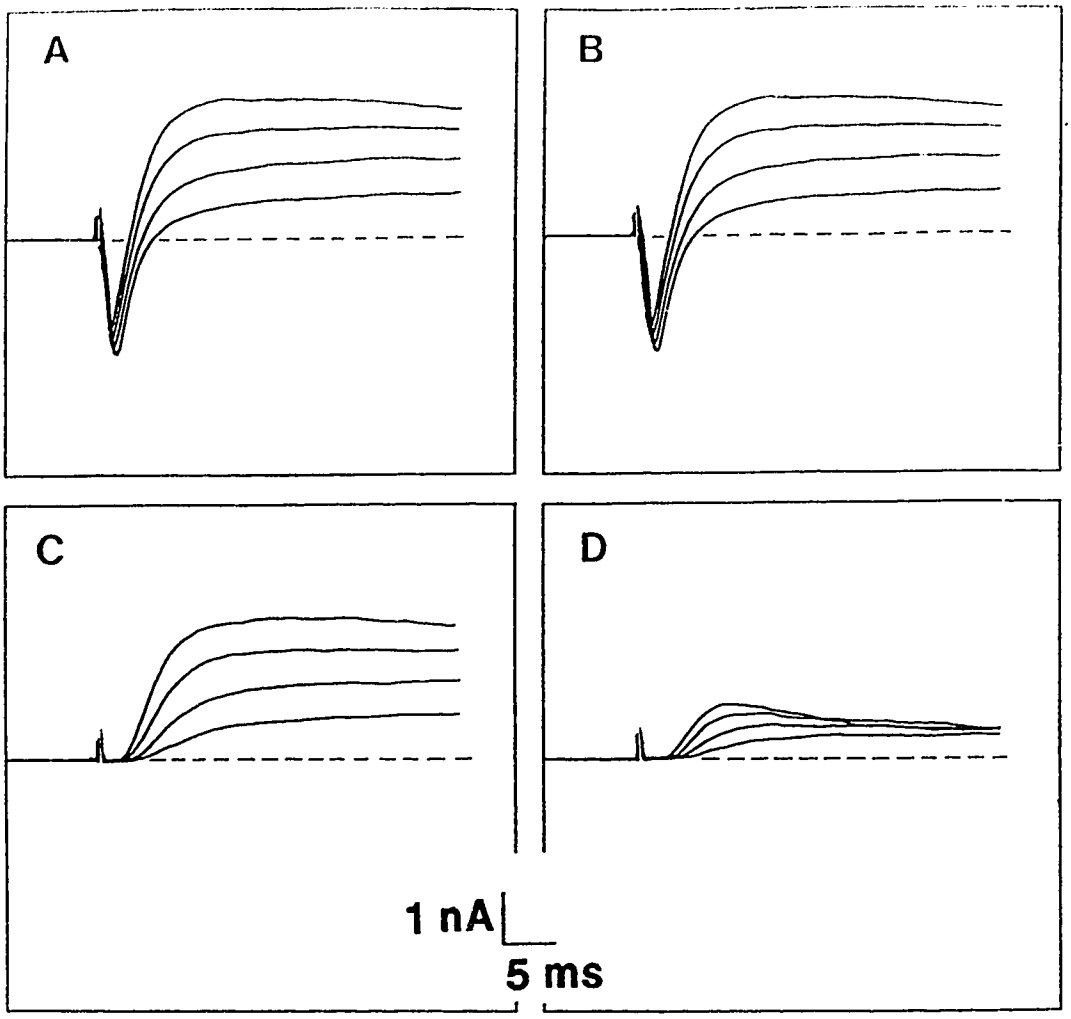

A
B
C
D
1 nA
5 ms

Fig.V-14. The effects of Bay K-8644/DMSO on Na and K channel currents in one N1E-115 cell

Fig.V-14. The effect of Bay K-8644/DMSO on Na^+ and K^+ channel currents in one N1E-115 cell. **A.** Control current traces. **B.** 5 min after the application of 10 μM Bay K-8644/DMSO. **C.** 5 min after application of 10 μM TTX. **D.** 10 min after the application of 10 mM TEA. Experiments shown in **A, B, C,** and **D** were carried out in the same cell. Holding potential was -80 mV. The membrane currents were elicited from -10 to +20 mV. Leakage and capacitive currents were subtracted. The composition of recording solutions has been described in Materials and Methods.

CHAPTER 6

Summary and Discussion

6.1. Summary

Dihydropyridines (DHPs) have been used extensively in the study of voltage-dependent calcium channels (VDCC). Small changes in the structure of DHPs in some cases result in great variations in their effects on VDCC. The actions of DHPs may be modified when dissolved in various solvents. In this study, the whole cell version of the patch clamp technique was used to investigate effects of nifedipine, a DHP L channel antagonist, and Bay K-8644, a DHP L channel agonist, dissolved in different solvents, on the two types of calcium channel currents in neuroblastoma cells (N1E-115). The concentration of all four solvents, dimethylsulfoxide (DMSO), acetone, ethanol (EtOH) and polyethylene glycol (PEG), was always fixed at 0.3% in the bath to reach different final concentrations of DHPs. At this fixed solvent concentration, the solvents alone did not have any effect on the transient (T) and the long-lasting (L) calcium channel currents. Both nifedipine and Bay K-8644 dissolved in DMSO decreased T channel currents. This inhibitory effect of DHPs dissolved in DMSO on T channel currents was dose-dependent and reversible. In contrast, T

channel currents were not inhibited by nifedipine dissolved in acetone or ethanol. In addition, Bay K-8644 dissolved in EtOH or PEG did not inhibit T channel currents. Pretreatment of cells with DMSO did not render the T channel current sensitive to Bay K-8644 dissolved in EtOH or PEG, or to nifedipine dissolved in acetone or EtOH. Dried nifedipine/DMSO or Bay K-8644/DMSO powder re-dissolved in acetone, EtOH or PEG were capable of inhibiting T channel currents. Furthermore, within the concentration range of 10 nM to 100 μM, nifedipine/DMSO inhibited L channel currents more effectively than did nifedipine dissolved in EtOH or acetone. Bay K-8644 (10 nM) increased L channel currents by 80%, but only 30% with DMSO and EtOH as the solvent, respectively. It is concluded that using DMSO as the solvent, nifedipine and Bay K-8644 not only act more effectively on L channels but also block T channels in neuroblastoma cells.

6.2. Discussion

6.2.1. The specific inhibitory effect of DHPs dissolved in DMSO on T channel currents

The concept that DHP antagonists act specifically on L channels but have no effect on T channels has recently been challenged. For example, the inhibition of T channels by nifedipine has been reported by Akaike *et al.* (1989). Another two dihydropyridines, i.e. isradipine (Loirand *et al.*, 1989) and felodipine (Van Skiver *et*

al., 1989), have been shown to inhibit T channel currents. The mechanism for this dihydropyridine antagonist-sensitive inhibition of T channels has not, however, been clarified. The present data show that the action of DHP antagonists and agonists can be dramatically modified by solvents. When dissolved in DMSO, nifedipine and Bay K-8644 blocked the T channel current and increased their effects on L channel currents. This novel result observed first with neuroblastoma cells can be extended to other cell preparations. In neonatal rat ventricular myocytes, preliminary data (not included in this thesis) showed that nifedipine/DMSO inhibited both T and L channel currents, and that Bay K-8644/DMSO inhibited T channel but increased L channel currents. However, when either acetone or ethanol were used to dissolve these two DHPs, only the modulation of L channel currents could be recorded in the presence of nifedipine or Bay K-8644. Other preliminary results indicate that Bay K-8644/DMSO, but not Bay K-8644/EtOH, blocked T channel currents in osteogenic sarcoma cells (Li Bing, personal communication). These results suggest that the solvent effect on the action of DHPs on T channels is not restricted to only a special neuronal preparation but is a general phenomenon. A more complete investigation on the solvent effects on the action of DHPs in cardiac myocytes and other cell preparations is required.

Concentrations ranging from 1 μM to 30 μM for nifedipine/DMSO and from 0.1 μM to 10 μM for Bay K-8644/DMSO were used in the present study to elicit a significant inhibition of T channel currents. Compared to the concentration of nifedipine used to inhibit L channel currents, this concentration of nifedipine/DMSO

is relatively high. Generally speaking, at higher concentrations DHPs can affect not only calcium channels but also other ionic channels. Yatani *et al.* (1988) have reported that nitrendipine/EtOH at a concentration of 10 μM inhibited Na channels in cardiac myocytes. In addition, (-)-Bay K-8644/EtOH at a concentration of 1 to 10 μM activated Na channels in neonatal rat cardiocytes (Kohlhardt *et al.*, 1989). This observation supports the report by Yatani *et al.* (1988). However, the inward rectifying, and the outward potassium channels were not affected by DHPs even at a concentration of 10 μM in cardiac myocytes (Yatani *et al.*, 1988). The possibility exists that nifedipine and Bay K-8644 dissolved in DMSO induced inhibition of T channel currents by a high-dose-evoked nonspecific action. The results described in this thesis indicate that this possibility is very unlikely for the following reasons. *1) Tissue-selectivity of DHP effect*: L type calcium channels are very sensitive to DHPs in cardiac (Sanquinetti *et al.*, 1984a) and vascular smooth muscle cells (Bean *et al.*, 1986). L channel currents were almost completely eliminated by 3 μM nitrendipine/PEG (Bean *et al.*, 1986). In contrast, in many neurons DHP antagonists have little effect on electrophysiologically identified L channels. For example, in bullfrog and rat DRG neurons, 3 μM nimodipine (solvent not indicated) blocked L channel currents by less than 50% (Bean, 1989). Nifedipine/EtOH at concentrations of 1 to 5 μM reduced L channel currents by 40% in chick DRG neurons (Rane *et al.*, 1987). This tissue selectivity of the DHP effect indicates a low sensitivity of neuronal calcium channels to DHPs or the existence of a non-L high-voltage-activated VDCC (Regan *et al.*, 1991). *2) Calcium channel subtype-selectivity of the*

DHP effect: Nifedipine or Bay K-8644 dissolved in acetone or EtOH had no effect on T channel currents at the same concentration range as nifedipine or Bay K-8644 in DMSO. This evidence argues strongly against the suggestion that the effect is due to a high-dose evoked non-selective action of DHPs. Moreover, L channel currents should be inhibited as well if the inhibition of T channel currents by Bay K-8644/DMSO were non-selective. However, Bay K-8644/DMSO decreased or increased the amplitudes of T and L channel currents, respectively. *3) Resistance of $Na^{\pm}$ and $K^{\pm}$ channel currents to DHPs*: In the present study, at a concentration as high as 10 μM, no effect on Na^+ and K^+ channel currents could be induced by DHPs/DMSO. Taken together, the conclusion is that the inhibition of T type calcium channel currents by Bay K-8644/DMSO and nifedipine/DMSO is a specific effect.

6.2.2. Comparison of the effects of nifedipine/DMSO and Bay K-8644/DMSO on T and L channel currents

Nifedipine/DMSO and Bay K-8644/DMSO have very similar effects on calcium channel currents. These two agents 1) inhibited T channel currents in a dose-dependent and reversible manner; but 2) did not shift the peak of the I-V relationship of T channels or change the kinetics of T channel currents; and 3) showed stronger effects on L channel currents than when these two agents were dissolved in other solvents. When dissolved in acetone or ethanol, both nifedipine and Bay K-8644 had no effect on T channel currents.

The similarities of the effects when comparing nifedipine/DMSO and Bay K-8644/DMSO indicate that a part of their structure will be affected similarly so that both of these two agents can affect T channels in the same way.

However, many differences also exist in their effects on calcium channels. 1) The nifedipine/DMSO-induced inhibition of the T channel current is dependent on the conditioning voltage. In the presence of nifedipine/DMSO, the steady-state inactivation curve shifted towards more negative potentials, suggesting that the binding of nifedipine to the inactivated state had a higher affinity than the resting state of the channel (Bean, 1984). In contrast, the inhibitory effect of Bay K-8644/DMSO on T channel currents was not potential-dependent. 2) Nifedipine/DMSO blocked both T and L channels. Bay K-8644/DMSO blocked T but amplified L channels. 3) Nifedipine/PEG inhibited T channel currents but Bay K-8644/PEG did not. 4) The concentration for half-maximal inhibition of T channel currents by nifedipine/DMSO or Bay K-8644/DMSO is approximately 10 μM or 0.6 μM, respectively. 5) Although the Bay K-8644 used consisted of a mixture of two optical isomers, nifedipine did not.

These differences in the effects of nifedipine and Bay K-8644 dissolved in various solvents indicated that an unidentified part in the structure of nifedipine and Bay K-8644 determined their specific actions on calcium channels when dissolved in different solvents. It is also possible that solvents have different effects on different DHPs.

6.2.3. The direct action of DMSO the DHP molecules

Solvents alone may have effects on VDCC (see Chapter 1). Once the functional state of the calcium channel is modified by solvents, the Ca^{++} channel becomes sensitive or more sensitive to nifedipine or Bay K-8644. In addition, long-term treatment of cells with some organic solvents will change the expression of specific types of calcium channels. DMSO has been reported to facilitate the differentiation of neuroblastoma cells (Narahashi *et al.*, 1987) and HL-60 cells (Baud *et al.*, 1987). After culture of N1E-115 cells for 1 month in media containing 2% DMSO, the T channel currents were no longer detectable, but the L channel currents could be recorded in these cells (Pang *et al.*, 1990). The following question was then posed: is it possible in the present study that the functional state of the T channel in the cell membrane was modified by DMSO, and hence the T channel became sensitive to nifedipine and Bay K-8644? In this study, the low concentration of DMSO (0.3%) and the short incubation time (<30 min) did not affect the T channel current. In addition, pretreatment of cells with DMSO did not render the cells susceptible to nifedipine/acetone or Bay K-8644/ethanol, nor did pretreatment of cells with acetone or ethanol change the action of Bay K-8644/DMSO or nifedipine/DMSO. Other solvents (acetone, PEG or EtOH) alone also had no effect on T and L channels currents at the concentrations used in this study. With a fixed DMSO concentration in the bath (0.3%), the inhibition of T channel currents by nifedipine/DMSO or Bay K-8644/DMSO was dose-dependent. This inhibition could be fully reversed by a 5 min wash-out with DHPs/DMSO-free bath solution. This

evidence did not support the possibility that solvents directly affected the VDCC in the present study. At high concentrations of DMSO, such as 20% (v/v), this solvent will dissolve the lipid bilayer and result in a non-selective increase in the membrane permeability (Rahaminoff *et al.*, 1988). Under some pathological conditions (i.e. hypoxia), the direct effect of DMSO on the cell membrane was to increase the calcium permeability (Barrett *et al.*, 1986). Based on the reports of Rahamimoff *et al.* (1988) and Barrett *et al.* (1986), nifedipine/DMSO in this study should increase the T channel currents rather than decrease them if the nifedipine/DMSO effect on T channel currents was purely a result of the membrane-dissolving effect of DMSO.

DHPs have been demonstrated to cross the lipid membrane without any restriction (Rhodes *et al.*, 1985). It may be hypothesized that DMSO can facilitate the passage of DHPs across the membrane more easily than acetone or ethanol. Therefore, the inhibitory effects on T channel currents by nifedipine/DMSO or Bay K-8644/DMSO may arise from the binding of the DHP on the intracellular side of the T channel protein. The effect of the intracellular application of DHPs on calcium channels in neurons has not been reported. However, it has been reported that in single cardiac cells nifedipine and nicardipine (1 to 10 μM), when included in the pipette solution to dialyse into the cell, increased L channel currents (Iijima *et al.*, 1984). Using planar lipid bilayers in which the DHP can be easily added to either the internal or external side of the membrane, Valdivia and Coronado (1990) investigated the sidedness of DHP effects on calcium channels in skeletal muscle cells. Nifedipine and (+)-Bay K-8644, being the calcium channel antagonists, applied

externally, behaved as calcium channel antagonists. However, when nifedipine and (+)-Bay K-8644 were applied to the cytosolic side, they were 2 to 10 times more effective than those applied externally (Valdivia and Coronado, 1990). A few questions remain unanswered in the above study. One of them is the state of the calcium channels used in that investigation. In addition, the calcium channels studied in the report of Valdivia and Coronado (1990) appear to be of the L type although there was no clear-cut designation. The sidedness of the effect of DHPs on T channels, therefore, is not clear from the study of Valdivia and Coronado (1990). Nevertheless, it is important that this study indicated that two DHP receptors were located on opposite sides of the membrane. They were coupled to, and modulated, the same calcium channels. In this study, dried nifedipine/DMSO or Bay K-8644/DMSO inhibited T channel currents even when the dried DHP powder was redissolved in acetone or ethanol. The point brought out by this experimental manoeuvre is that the DHP effect on T channels is not due to a better facilitation of the transportation of DHPs induced by DMSO but that DMSO affected the conformation of the DHP molecules. Once this conformational change occurred, the DHP was able to bind to its receptor(s) independent of the solvents used.

Whenever a solvent is involved in the effect of a specific agent the first question is the solubility of the agent. If the solubility of nifedipine is poor in acetone or ethanol, the effective concentration of nifedipine reaching the cell will be too low to affect T channel currents. This is true when water is used as the solvent, but is not the case in this study. Within the concentration range used in the present

study, both nifedipine and Bay K-8644 were well dissolved in acetone, ethanol, PEG or DMSO. Also, when the concentrations of nifedipine and Bay K-8644 dissolved in acetone or ethanol were increased from 30 or 10 μM to 100 μM, the T channel current was not affected. This observation does not fit the hypothesis that a lower concentration of nifedipine or Bay K-8644 was obtained when these substances were dissolved in acetone or ethanol. The next possibility which is related to DHP solubility is the potential precipitation of DHPs when the stock solution of the DHP was added to the bath solution. This would explain the lack of inhibition of T channel currents by DHPs dissolved in acetone or ethanol as an artifact caused by precipitation of the agent when added to the bath. Ultraviolet absorbance measurements revealed a linear relationship between the maximum absorbance and the concentrations of nifedipine or Bay K-8644 within the concentration range tested. If any precipitation of nifedipine or Bay K-8644 occurred in the mixture of stock solution with bath solution at the concentrations used in these experiments, the plot of absorbance against DHP concentrations would show saturation.

A possible explanation regarding the inhibition of T channel currents and the more effective actions on L channel currents produced by DHPs is that this effect may be due to the direct interaction of DMSO and DHP molecules. DMSO may change the molecular conformation of DHPs in some unknown way. It is possible that DHPs have an intrinsic capacity for binding and modulating both T and L channels. Some solvents may change or break down the molecules of the DHPs so that the DHPs cannot bind to T channels, whereas other solvents do not. This may

explain the inability of nifedipine/acetone to affect T channels. Nishi *et al.* (1983) used nifedipine·HCl dissolved in bath solution and did not report an inhibitory effect on the T channel current in helix neurons. Since nifedipine·HCl is readily soluble in physiological saline without organic solvents, a molecular change is not expected. Hence, the study by Nishi *et al.* (1983) strongly supported the idea that acetone or ethanol did not modify the DHP molecule. Furthermore, dried Bay K-8644/DMSO or nifedipine/DMSO powder that was re-dissolved in EtOH or PEG inhibited T channel currents. This result can be interpreted to mean that the molecular change induced in the DHP molecule when it was first dissolved in DMSO was permanent. When the DHP was subsequently re-dissolved in acetone or ethanol, there was no additional change in the conformation of DHP molecule. If the molecular change is sufficient, UV absorbance spectra may detect some variation at shorter wavelengths. However, the full UV absorbance spectra was not examined in the present study. This possibility cannot be fully excluded. The data in this study show that dried Bay K-8644/DMSO or nifedipine/DMSO re-dissolved in DMSO had the same potency as Bay K-8644/DMSO or nifedipine/DMSO. Hence, the change in the DHP molecule was apparently not due to the drying process. A further possibility is that optical forms of DHPs are modified by the solvents. Franckowiak *et al.* (1985) used isolated rabbit aortic and heart preparations to study the effect of optical isomers of Bay K-8644 dissolved in DMSO. They found that (-)Bay K-8644 exhibited the known vasoconstricting and positive inotropic properties of the Ca^{++} agonistic compound, while its antipode (+)Bay K-8644 (10-50 times higher concentration)

exhibited the vasodilating and negative inotropic effects of Ca^{++} antagonistic drugs. Using the patch-clamp technique, Kass (1987) reported that (+)Bay K-8644 (Bay K-4427) dissolved in PEG inhibited the L channel current, while (-)Bay K-8644 (Bay K-5417) enhanced or inhibited L channel currents and that this inhibition depended on the holding potential. If Bay K-8644, i.e. (±) Bay K-8644, dissolved in DMSO became (+)Bay K-8644 (one of the isomers of (±)Bay K-8644), this derivative may have a different binding affinity and function from those of (±)Bay K-8644 on T and L channels. In fact, McCarthy and Cohen (1986) have suggested that (+)Bay K-8644 might inhibit the T channel current in GH_3 cells. Due to the difficulty in obtaining the two optical isomers of Bay K-8644, this possibility was not examined in the present experiments. Nevertheless, it is not a likely possibility since Bay K-8644/DMSO still enhanced L channel currents while (+) Bay K-8644 would be expected to inhibit L channel currents. Furthermore, the nifedipine used in this study was not a racemic mixture, but nifedipine/DMSO also blocked T channel currents. Hence, the solvent effects on the action of DHPs cannot be ascribed to an isomer shift in the case of those DHPs which are used as a racemic mixture. An interesting possibility is that different solvents may exert various effects on the ring distortion of the DHPs. The most active compounds showed the smallest degree of ring distortion from planarity (Fossheim, 1986). This question can be addressed using modern spectroscopic methods. The nuclear magnetic resonance (NMR) method can be used to measure nuclear spin coupling with an applied magnetic field. Electron-spin resonance is usually used to map unpaired-electron distribution in

molecules and molecular fragments. Ultraviolet-Visible (UV) spectroscopy is able to detect the electron transitions in molecules and atoms. However, the most effective and suitable method to find minor rotation and/or vibration of a molecule is far-infrared spectroscopy (Bradley, 1978). Further studies using this technique may suggest clues regarding the ring distortion and rotation of DHPs in the presence of different solvents (Janis and Triggle, 1983; Fossheim, 1986).

6.2.4. The homogeneity of T and L channels - An indication derived from the effects of DHPs

Along with their differences, T and L channels are similar in many aspects. Their steep voltage dependence and high selectivity for calcium over sodium show a great degree of similarity. Both T and L channels possess multiple high-affinity divalent binding sites in their permeation pathway (Carbon and Lux, 1987), while different intra-channel binding sites would be responsible for the difference in the ionic selectivity between T and L channels. Pharmacologically, subtypes of VDCC share some common features, as well. It is well known that ω-conotoxin preferentially blocks N channels. However, this toxin also has a weak but reversible inhibitory effect on T channels in cardiac and neuronal cells (Oyama *et al.*, 1987; Glossmann and Striessnig, 1990).

Yoshii *et al.* (1988) reported that type I (T) and type II (L) calcium channels had similar properties in their gating and permeation mechanisms despite their differential voltage sensitivity and ionic selectivity. Hirano and Takahashi (1984)

investigated membrane excitability in *Ascidians* embryos and found that the calcium channels emerging at early stages of development were T channels, while those which developed at later stages were L channels. Pang *et al.* (1990) found that after complete differentiation, T channel currents were no longer detectable, and L channel currents emerged as the predominant currents in neuroblastoma cells. These studies suggested that the origins of T and L channels may be the same. Using DMSO as the solvent, both nifedipine and Bay K-8644 (Wu *et al.*, 1990) can affect T and L channels. Na and K channel currents were not affected. The results of this study reflect the prediction made by Bean that "T channels may well be more sensitive to dihydropyridines than most non-Ca channels" (1990).

Comparing these similarities between T and L channels (even including N channels), it is very tempting to postulate that these subtypes of VDCC may belong to the same gene family. Due to the lack of specific ligands, the purification of T channels depends only on cloning and/or expression techniques. The expression of T and L types of VDCC in *Xenopus* oocytes after injection of total mRNA from rat heart and brain has been achieved (Dascal *et al.*, 1986). The total mRNA injected in the above studies may represent an ancient gene, and T and L subtypes may be the splitting products of the same gene origin. The structural necessity for DHPs/DMSO to inhibit T channels may represent similar DHP binding domains for both T and L channel proteins, which further suggests the structural homology of T and L channels.

Using radioactive 1,4 DHPs, the amino acid sequence of many essential

subunits of L channels have been identified. Expression of functional L channel currents with mRNA specific for transcription of the cardiac α_1 subunit or skeletal muscle α_1 subunit has been reported in *Xenopus* oocytes (Mikami *et al.*, 1989) and murine L-cells (Perez-Reyes *et al.*, 1989). Total mRNA for T and L channels has been successfully isolated (Dascal *et al.*, 1986), hence the cloned T channel cDNAs will soon be available. In the present study, the DHP-sensitivity of T channels has been established and traced to the solvent effect. Taken together all this important progress in the understanding of the homology of subtypes of VDCC will eventually produce a clear picture of the structural similarity between T and L channel proteins.

6.2.5. Significance of the study

One of the important contributions of the present study is that it provides direct electrophysiological evidence that dihydropyridines can modulate T type voltage-dependent calcium channels and that this effect depends on the solvent. Without taking the solvent effect into account it is difficult to provide definite pharmacological evidence for the separation of T and L channels. Moreover, this study will help us to understand the molecular conformation of VDCC and may provide important tools to purify not only L-, but also T-type calcium channel proteins. Finally, the results from the present investigation will eventually facilitate the design of more effective and more selective calcium channel antagonists and agonists.

Table-1. Summary of the effects of DHPs dissolved in different solvents on the T and L channel currents in N1E-115 cells

Drug	Solvent	T	L
Nifedipine	DMSO	- -	- -
	PEG	-	-
	EtOH	no effect	-
	acetone	no effect	-
Bay K-8644	DMSO	- -	+ +
	PEG	no effect	+
	EtOH	no effect	+

Note: -, and + indicate an inhibitory effect and an excitatory effect, respectively.

CHAPTER 7

Conclusions

Dihydropyridines are important agents used for probing the structure and function of calcium channels in a variety of tissues. Classically, these agents have been thought to bind selectively to L-type calcium channels with nifedipine as an antagonist and Bay K-8644 as an agonist. However, this traditional concept has been challenged recently. Since DHPs are practically insoluble in water, they are most commonly dissolved in organic solvents, such as ethanol, polyethylene glycol, acetone or DMSO. The modification of the effects of DHPs on voltage-dependent calcium channels by different solvents has not, however, been carefully and systemically studied.

In the present study, the whole-cell version of the patch clamp technique was used to investigate the effects of nifedipine and Bay K-8644, dissolved in different solvents, on T- and L-type voltage-dependent calcium channels in neuroblastoma cells (N1E-115). Within the concentration range studied, solvents alone did not exhibit any effect on T and L channel currents. Nifedipine dissolved in DMSO decreased the T channel current. This decrease was concentration-dependent, potential-dependent and reversible. Nifedipine dissolved in acetone or ethanol had no such effect on T channels. Dried nifedipine/DMSO powder re-dissolved in

acetone or ethanol, however, could inhibit the T channel current. Furthermore, nifedipine/DMSO inhibited the L channel current more effectively than did nifedipine dissolved in ethanol or acetone. Correspondingly, it was demonstrated that with DMSO as the solvent, Bay K-8644 inhibited the T channel current, while Bay K-8644 dissolved in ethanol or PEG had no effect on the T channel current in neuroblastoma cells. The inhibitory effect of Bay K-8644 on T channel currents depended on drug concentration and disappeared after washout with drug-free bath solution. DMSO pretreatment of cells did not render the T channel current sensitive to Bay K-8644 dissolved in ethanol or PEG. Dried Bay K-8644/DMSO powder re-dissolved in ethanol or PEG inhibited the T channel current. In addition, Bay K-8644 increased L channel currents more effectively than did Bay K-8644 dissolved in ethanol.

It is concluded that, via a direct interaction between the solvent and DHPs, nifedipine and Bay K-8644 dissolved in DMSO affect both L and T channel currents in N1E-115 cells. The results from the present study will have a profound impact on studies of the mechanisms of action of DHPs, and the experimental and clinical application of DHPs. In addition, the present data will lead to a more critical evaluation of the pharmacological criteria which separate the T and L channels, and will help to elucidate the molecular structure of T and L channels.

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