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THREE TYPES OF VOLTAGE-DEPENDENT CALCIUM CURRENTS DEVELOPING IN CULTURED HUMAN NEUROBLASTOMA CELLS

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ABSTRACT

Voltage-dependent calcium currents (I_{Ca}), which developed in cultured human neuroblastoma cells (NB-I), were studied using a whole-cell recording technique. Three types (T-, N- and L-type) of I_{Ca} were identified based on their biological and pharmacological properties. The T-type I_{Ca} was observed in about 60% of the cells from day 2 to day 12 and in about 20% after day 14. Likewise, the amplitude gradually decreased from –61.7 ± 10.1 pA on day 2 to –18.3 ± 9.1 pA on day 18. The N-type I_{Ca} appeared on day 6, with the number of cells exhibiting this current increasing up to 90.9% (–73.4 ± 16.0 pA) on day 14 and immediately decreasing thereafter. The L-type I_{Ca} was observed in 50.0% of the cells on day 2, increasing to 84.6% of the cells on day 6 and remaining thereafter, while the amplitude gradually increased from –37.8 ± 14.0 pA on day 2 to –158.8 ± 22.8 pA on day 18, and decreased thereafter. These findings indicate that the expression of the voltage-dependent Ca^{2+} channels is strongly regulated by the developmental stage of the cell.

Key Words: Calcium channel current, Whole cell recording, Human neuroblastoma NB-I, Development

INTRODUCTION

Voltage-dependent Ca^{2+} channels play important roles in the regulation of many cellular functions.[1] Recent studies suggest that the expression of the voltage-dependent Ca^{2+} channels is strongly regulated by the developmental stage of the cell.[2] Three types (T-, N- and L-type) of Ca^{2+} current (I_{Ca}) were found to be present in human neuroblastoma cells.[3,4] In this study we describe the developmental changes, i.e., 1) frequency and 2) amplitude changes, in the expression of the three types of I_{Ca} (T-, N- and L-type I_{Ca}) in neuroblastoma NB-I[5] cells of human origin.

MATERIALS AND METHODS

Details of the cell culture, recording conditions, and solution were as described previously.[3,6] Briefly, the human neuroblastoma cell line (NB-I) established by Miyake et al. was used.[5] Cells were cultured in RPMI 1640 medium, pH 7.4, supplemented with 10% fetal calf serum at a temperature of 37°C. NB-I cells were treated with 0.25% trypsin and were placed on a small

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glass-covered culture dish and incubated for 2 to 22 days before use. Since cells become extinct due to multiplication, cells were placed subcofluently and some cells were periodically dumped. The day on which the cells were treated with trypsin was defined as day 0.

A whole-cell recording patch-clamp technique was used to record the I_{Ca} of the neuroblastoma cells.[7] I_{Ca} as evoked by applying voltage steps of 400 ms in duration from –100 mV to + 80 mV at 10 mV steps from the holding potential. The holding potentials were set at –80 mV for T- and N-type I_{Ca} and at –30 mV for the L- type I_{Ca}. Experiments were carried out at room temperature (22–25°C).

The normal external solution contained (in mM): NaCl (36.7), $BaCl_2$ (51.2), $MgCl_2$ (1.18), glucose (11.8), HEPES-Na (10.0), tetraethylammonium-Cl (TEA) (23.6), and tetrodotoxin (TTX) 3×10^{-3} at pH 7.4. Patch pipettes (3 to 5 MΩ) were filled with a solution containing (in mM): Cs-Aspartate (106.2), CsCl (23.6), $MgCl_2$ (4.95), ATP-Na_2 (4.95), EGTA (9.9), HEPES-Na (4.95), and $CaCl_2$ (1.26) at pH 7.0. Ba^{2+} was used in the external solution because the Ca^{2+} is more permeable to Ba^{2+} than Ca^{2+} in solution, thereby making it easier to analyze the current's amplitude.[7] We studied the effects of some inorganic blockers (Ni^{2+}, Cd^{2+} and La^{3+}), the dihydropyridine Ca^{2+} channel blocker nifedipine, ω-CgTX[8], and Bay K 8644[9] on the different types of I_{Ca} in NB-I cells.

Membrane currents were recorded using a CEZ 2300 pre-almplifier (Nihon Kohden, Tokyo, Japan). Data were analyzed using a software package (PCLAMP ver. 5.51, Axopatch, USA). The amplitude of the N-type I_{Ca} was measured by Nowycky's method.[10] The experiments were carried out every other day from day 2 to day 22 of culture. Data were expressed as mean values ± standard mean error.

RESULTS

The electrophysiological and pharmacological properties of the three types of I_{Ca} (T, N, L-type) have been described previously.[4,6] Figure 1 shows the three types of I_{Ca} recorded in NB-I cells. Figure 1-A shows the time course of the I_{Ca} evoked by a depolarizing stimulation of 400 ms duration. Figure 1-B shows the current-voltage relationship (I-V curve). The T-type I_{Ca} was activated by a depolarizing potential higher than –50 mV, and was rapidly inactivated during the application of depolarizing test potentials of –10 mV with a time constant of 22.5 ± 5.7 ms (n = 4) (Fig. 1-A-a and Fig. 1-B-a). The N-type I_{Ca} was activated at a relatively large depolarization potential higher than –20 mV, and decayed with a time constant of 120 ± 8.8 ms (n = 5) at a test potential of 20 mV (Fig. 1-A-b and Fig. 1-B-b). The L-type I_{Ca} was activated at depolarization potentials (Vt higher than 0 mV) and showed little inactivation during a 400 ms depolarization (Fig. 1-A-c and Fig. 1-B-c). The voltage dependency of the inactivation of the T, N-, and L-type I_{Ca} was evaluated. The T-type I_{Ca} was strongly inactivated between –80 mV and –40 mV, and was completely inactivated upon the application of a –30 mV prepulse. The data points for the T-type I_{Ca} were fitted by a continuous smooth curve derived from the Boltzmann equation with a mid-point of -64 mV and a slope parameter of 4.1 mV (n = 5). The data points for the N-type I_{Ca} were fitted by a smooth curve derived from the Boltzmann equation with a mid-point of –32 mV and a slope parameter of 10.9 mV (n = 7). The data points for the L-type I_{Ca} were also fitted by the Boltzmann equation with a mid-point of –18 mV and a slope parameter of 8.3 mV (n = 4).

In table 1, the electrophysiological and pharmacological properties of the three types of I_{Ca} (T, N, and L-type) recorded in NB-I cells are listed. Relative conductances were measured when recordings were made using a 10 mM external Ca^{2+}, instead of a 50 mM external Ba^{2+},

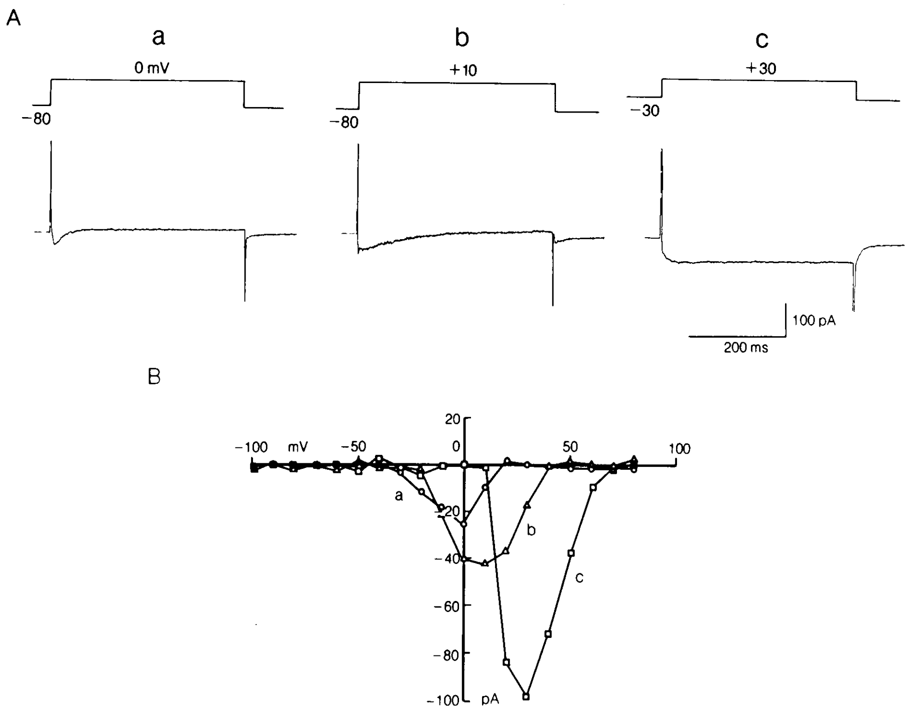

Fig. 1: Voltage-dependent I_{Ca} in the cells of the human neuroblastoma (NB-I) cell line.
(A) Typical inward currents evoked by applying step depolarization from a holding potential of –80 mV for T-type I_{Ca} (a), N-type I_{Ca} (b), and of –30 mV for L-type I_{Ca} (c) to the testpotentials indicated.
(B) The typical current-voltage relationships of T-type I_{Ca} (a), N-type I_{Ca} (b), and L-type I_{Ca} (c).
○: T-typpe I_{Ca} △: N-type I_{Ca} □: L-type I_{Ca} Data were individually obtained from different cells.

solution. Extracellular application of 100μM Ni^{2+} inhibited the T-type I_{Ca} by 82.6% (n = 8). On the other hand, 100 μM Cd^{2+} inhibited the N-type I_{Ca} and L-type I_{Ca} by 90.5% (n = 3) and 97.0% (n = 3), respectively. La^{3+} at 10μM inhibited the L-type I_{Ca} by 95.8% (n = 3). Nifedipine at 10μM inhibited the L-type I_{Ca} by 90.1% (n = 3). ω-CgTX at 5μM inhibited N-type I_{Ca} by 66.6% (n = 4). Bay K 8644 at 10 μM, an L-type Ca^{2+} channel agonist,[8] enhanced the L-type I_{Ca} by 32.4% (n = 9) when compared to the control state.

Figure 2 shows the appearance of the three types of I_{Ca}. The T-type I_{Ca} appeared in 66.7% of cells on day 2, about 60% of cells from day 4 to day 12, in 27.3% of cells on day 14, and about 20% after day 16 of culture in the examined cells. The N-type I_{Ca} first appeared in 7.7% on day 6, with the number of cells exhibiting this current increasing up to 90.9% on day 14 in the examined cells and immediately decreasing thereafter. The L-type I_{Ca} appeared in 50.0% of cells on day 2, increasing to 84.6% of cells on day 6 in the examined cells and remained thereafter.

Figure 3 shows the amplitude development of the three types of I_{Ca}. The amplitude of the T-type I_{Ca} gradually decreased from –61.7 ± 10.1 pA on day 2 to –18.3 ± 9.1 pA on day 18.

Table 1: Electrical and pharmacological properties of the three types of I_{Ca} in human neuroblastoma cell line NB-I cells. Each value is the average ± S.E.M. of 5-8 cells.

	T-type I_{Ca}	N-type I_{Ca}	L-type I_{Ca}
Acitivation range (for 50mM Ba^{2+})	> −50mV	> −20mV	> 0 mV
Inactivation rate (τ : ms) (50mM Ba^{2+})	22.5±5.7 (−10mV)	120.4±8.8 (20mV)	>400
Relative conductances (Ca^{2+}/Ba^{2+})	1.02	0.68	0.17
Ni^{2+} (100 μM) inhibition	82.6±15.3 %	38.7±18.8 %	69.6±6.8 %
Cd^{2+} (100 μM) inhibition	11.4±3.2 %	90.5±0.5 %	97.0±0.4 %
La^{3+} (10 μM) inhibition	24.3±5.5 %	66.7±16.7 %	95.8±0.9 %
Nifedipine (10 μM) inhibition	20.6±0.6 %	40.0±9.6 %	90.1±5.9 %
ω-CgTX (5 μM) inhibition	12.9±7.6 %	66.6±12.2 %	26.9±8.0 %
Bay K 8644 (10 μM) enhancement.	−5.8±18.6 %	−2.6±11.6 %	32.4±27.2 %

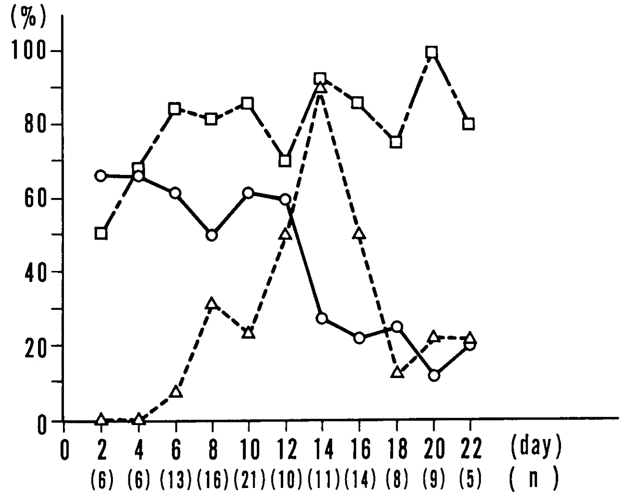

Fig. 2: Appearance of the three types of I_{Ca}
The T-type I_{Ca} was observed in about 60% of the cells examined from day 2 to day 12 with a sudden decrease to about 20% after day 14. The N-type I_{Ca} appeared on day 6, quickly increased to its peak, whereby it was observed in about 90.9% of the cells examined on day 14 and immediately decreased to about 20% thereafter. The L-type I_{Ca} were observed on day 2 in 50% of the cells examined and gradually increased to be present in 80% to 100% of the cells after day 6. ○: T-type I_{Ca} △: N-type I_{Ca} □: L-type I_{Ca}

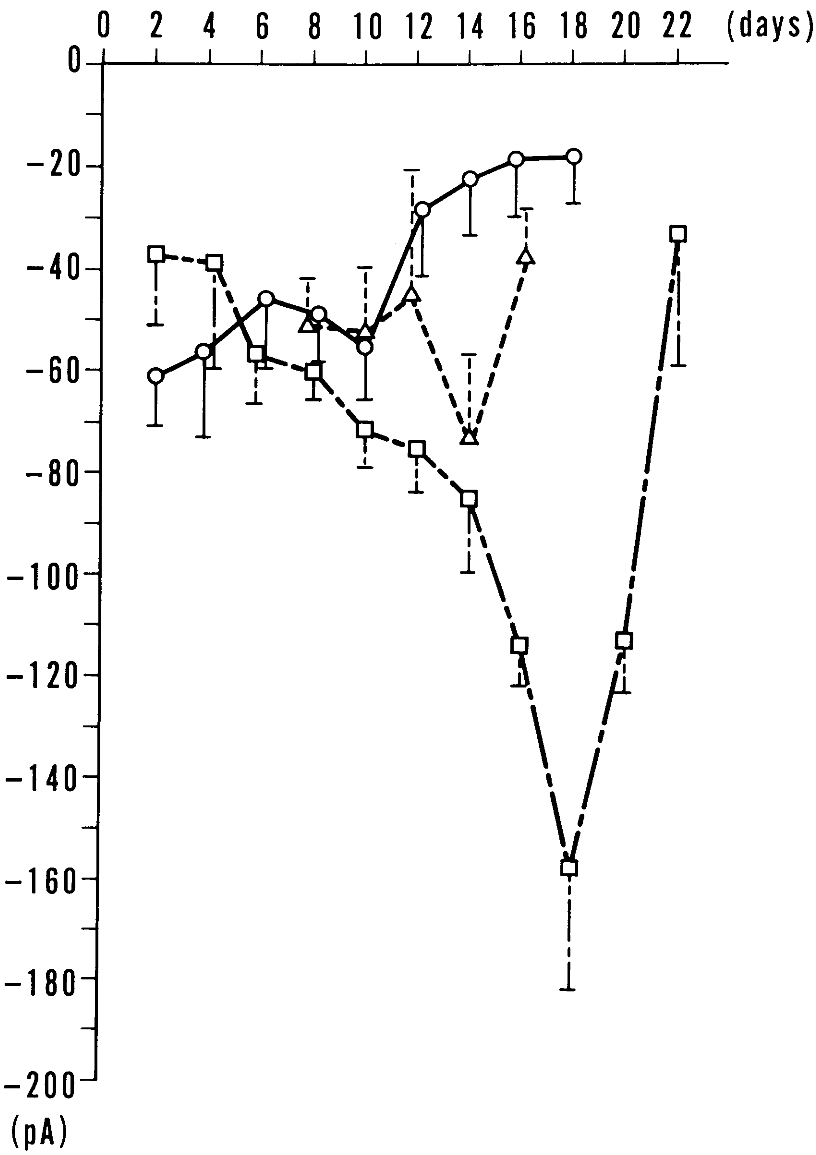

Fig. 3: Amplitude development of the three types of I_{Ca}
The amplitude of the T-type I_{Ca} was –61.7 ± 10.1 pA at day 2, gradually decreased at day 6, transiently increased to –55.7 ± 10.5 pA at day 10 and gradually decreased to –18.3 ± 9.1 pA at day 18. The N-type I_{Ca} appeared with an amplitude of –51.5 ± 9.0 pA at day 6, reached its peak of –73.4 ± 16.0 pA at day 14 and suddenly decreased to –38.6 ± 10.2 pA at day 16. The L-type I_{Ca} appeared with an amplitude of –37.8 ± 14.0 pA at day 2, gradually increased and reached its peak of –158.8 ± 22.8 pA at day 18 and unexpectedly decreased to –33.9 ± 25.7 pA at day 22. ○: T-type I_{Ca} △: N-type I_{Ca} □: L-type I_{Ca}

The amplitude of the N-type I_{Ca} reached its peak of –73.4 ± 16.0 pA on day 14 and immediately decreasing thereafter. The amplitude of the L-type I_{Ca} gradually increased from –37.8 ± 14.0 pA on day 2 to –158.8 ± 22.8 pA on day 18, and immediately decreased thereafter.

DISCUSSION

Ca^{2+} channels are important in the physiological function of neurons, but little is known about the individual role of each type of Ca^{2+} channel.[1] The T-type Ca^{2+} channel may be involved in near-threshold membrane potential. For example, it may speed up the rate of depolarization to the threshold potential after neuronal hyperpolarization. Suzuki et al. reported that T-type Ca^{2+} channels mediate the transition between tonic and phasic firing in thalamic neurons.[11] The T-type I_{Ca} may be involved in the generation of absence seizures.[6,12] In this study, the T-type I_{Ca} was activated from day 2 to day 10. Therefore, the NB-I cells may be easily depolarized at the beginning of culture. The N-type Ca^{2+} channels activate neurotransmitter release[13] and control neuronal migration.[14] In this study, the activity of N-type Ca^{2+} channels reached a peak at day 14. Therefore, neuronal migration may be completed and neurotransmitter release may start around day 14. The L-type Ca^{2+} channels are activated during the generation of action potentials. Since L-type Ca^{2+} channels are more involved in increasing intracellular Ca^{2+} concentration than are T- and N-type Ca^{2+} channels, they may influence long-term cell functions via the second messenger system and gene expression.[1,10] Ca^{2+} channel blockers are clinically used to treat cardiovascular diseases including cardiac arrhythmia and hypertension[15] and neurologic diseases such as migraine and epilepsy.[16] The onset of the functional plasticity of cells appears to be correlated with the development of L-type I_{Ca}.[2] In our study, the activity of the L-type I_{Ca} gradually increased and reached its peak at day 18. Therefore, L-type I_{Ca} activity may influence the function of gene expression and the second messenger system. Further studies are needed to clarify the relationship between the functional role and the development of the three types of I_{Ca} in the nervous system.

REFERENCES

1) Kennedy, M.B.: Regulation of neuronal function by calcium. *Trends Neurosci.*, 11, 417–424 (1989).
2) Galewski, S., Skangiel-Kramska, J., Pomorski, P. and Kossut, M.: Voltage-dependent L-type calcium channels in the development and plasticity of mouse barrel cortex. *Dev. Brain.Res.*, 67, 293–300 (1992).
3) Carbone, E., Sher, E. and Clementi, F.: Ca currents in human neuroblastoma IMR32 cells: kinetics, permeability and pharmacology. *Pflügers. Arch.*, 416, 170–179 (1990).
4) Kito, M., Maehara, M., and Watanabe, K.: Three types of voltage-dependent calcium currents in cultured human neuroblastoma cells. *Nagoya J. Med. Sci.*, 58, 29–33 (1995).
5) Miyake, S., Shimo, Y. and Kitamura, T.: Characteristics of continuous and functional cell line NB-1, derived from a human neuroblastoma. *J. Auton. Nerv. Syst.*, 10, 115–120 (1973).
6) Kito, M., Maehara, M., and Watanabe K.: Mechanisms of T-type calcium channel blockade by zonisamide. *Seizure*, 5, 115–119 (1996).
7) Hamil, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J.: Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers. Arch.*, 391, 85–100 (1981).
8) Kasai, H., Aosaki, T. and Fukuda, J.: Presynaptic Ca-antagonist ω-conotoxin irreversibly blocks N-type Ca-channels in chick sensory neurons. *Neurosci. Res.*, 4, 228–235 (1987).
9) Nowycky, M.C., Fox, A.P. and Tsien, R.W.: Long-opening mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. *Proc. Natl. Acad.Sci.* U.S.A., 82, 2178–2182 (1985).

10) Nowycky, M.C., Fox, A.P. and Tsien, R.W.: Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* (Lond.), 316, 440–443 (1985).
11) Suzuki, S. and Rogawski, M.A.: T-type calcium channels mediate the transition between tonic and phasic firing in thalamic neurons. *Proc. Natl. Acad. Sci.U.S.A.*, 86. 7228–7232 (1989).
12) Porter, R.J.: The absence epilepsies. *Epilepsia,* 34 (Suppl.3), 42–48 (1993).
13) Hirning, L.D., Fox, A.P., McClesky, E.W., Olivera, B.M., Thayer, S.A., Miller, R.J. and Tsien, R.W.: Dominant role of N-type Ca^{2+} channels in evoked release of norepinephrine from sympathetic neurons. *Science,* 239, 57–60 (1988).
14) Komuro, H. and Rakic, P.: Selective role of N-type calcium channels in neuronal migration. *Science*, 257, 806–809 (1992).
15) Vanhoutte, P.M. and Paoletti, R.: The WHO classification of calcium antagonists. *Trends Pharmacol. Sci.*, 8, 4–5 (1987).
16) Overweg, J., Binnie, C.D., Meijer, J.W.A., Meinardi, H., Nuijten, S.T.M., Schmaltz, S. and Wauquier, A.: A double blind placebo-controlled trial of flunarizine an add-on therapy in epilepsy. *Epilepsia*, 25, 217–222 (1984).