International Journal of Oral Biology, September 1998, p. 141~147 Vol. 23, No. 3


# Isolation of Medullary Dorsal Horn Neurons and Characteristics of Membrane Currents

Seog Bae Oh, Kyungpyo Park, Seong-Ah Kim, Jong-Heun Lee and Joong-Soo Kim†

*Department of Physiology and Oral Biology, College of Dentistry, Seoul National University*

**Medullary dorsal horn (MDH) neurons receive noxious inputs from primary afferent fibers in the orofacial area, integrate modulatory influences from descending and local circuits and send the information to the thalamus and somatosensory cortex. In addition, this region contains many putative neurotransmitters, including excitatory and inhibitory amino acids and peptides. Because of the importance of MDH neurons in orofacial pain transmission, the modulation of activities of these cells is very interesting. In this study, we tried to establish a method for isolating and preparing MDH neurons for the purpose of studying the pain modulation mechanism in future, and identify if the acutely isolated cells preserve their electrophysiological activities using patch-clamp and fura-2-based microfluorometry techniques. Transverse slices (400 μm) of lower brainstem, 2-3 mm caudal to the obex, from neonatal rats (1-2 weeks old) were sequentially digested with two proteases (pronase 1 mg/ 5 ml, and thermolysin 1 mg/5 ml) at 32°C for 20 min. The MDH region was then dissected out and mechanically dissociated under a stereomicroscope using a series of fire-polished glass pipettes with a variety of orifice sizes. Single isolated cells were used for the experiment. This method yields multipolar, bipolar, unipolar, ellipse and pyramidal cells. These cells seem to be marginal cells of lamina I, stalked cells or islet cells in lamina II and pyramidal cells in lamina III, respectively. Fura-2-based microfluorometry showed that the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) increased in response to the membrane depolarization induced by high $K^+$, and the increase of $[Ca^{2+}]_i$ was caused by the influx of extracellular $Ca^{2+}$, demonstrating the expression of voltage-sensitive calcium channels (VSCC) in MDH neurons. From whole-cell patch-clamp techniques, voltage-sensitive $Na^+$ and $Ca^{2+}$ currents were directly recorded, the results were similar to those studied in *in vivo* or slice preparations. These facts indicate that electrophysiological activities are well preserved in the acutely isolated neurons, and these cells can be effectively used for biophysical and pharmacological studies, including understanding the pain modulation mechanism mediated by VSCC in the MDH.**

***Key words :*** medullary dorsal horn, patch-clamp, microfluorometry, voltage-sensitive $Ca^{2+}$ channels

## Introduction

The medullary dorsal horn (MDH), or the subnucleus caudalis of the trigeminal spinal tract nucleus, is a major relay site for orofacial pain transmission (Sessle, 1989) and has anatomical and functional similarities with the spinal dorsal horn (Dubner *et al.*, 1978). MDH neurons receive noxious inputs from primary afferent fibers, integrate modulatory influences from descending and local circuits and send the information to the thalamus and somatosensory cortex (Willis and Coggeshall, 1991). In addition, this region contains many putative neurotransmitters, including excitatory and inhibitory amino acids and peptides (Sessle, 1987). Because of the importance of MDH neurons in orofacial pain transmission, the modulation of the activities of these cells is very interesting. However, the precise mechanism underlying the modulation of sensory transmission still remains to be fully elucidated. The heterogeneity of the cells and the complexity of their synaptic organization and interactions make such study difficult.

Even though the responses of MDH neurons have been widely investigated in *in vivo* preparations (Hu *et al.*, 1981; Sessle *et al.*, 1981), individual wide dynamic range neurons in *in vitro* preparations have not been extensively used for the electrophysiological investigation of the interaction among neurotransmitters related to pain modulation. A limitation to study is the fact that the patch clamp technique (Hamill *et al.*, 1981), which provides a powerful access to the electrical properties of cell membranes, can only be applied to clean surface membranes. Therefore, it requires developing a method for preparing isolated

†Correspondence to: Dr. Joong-Soo Kim, Department of Physiology and Oral Biology, College of Dentistry, Seoul National University, Seoul 110-749, Korea.

neurons (Kay and Wong, 1986). Recent studies *in vitro*, especially of the spinal dorsal horn (DH), have shown that isolated dorsal horn neurons using both enzymatic and mechanical dissociation, can be useful in elucidating the cellular mechanisms underlying pain modulation (Cerne *et al.*, 1993; Kolaj *et al.*, 1995; Randic *et al.*, 1990; Rusin and Randic, 1991; Rusin *et al.*, 1993; Wang *et al.*, 1994). The use of acutely isolated DH neorons has permitted analysis of direct postsynaptic interactions between excitatory neurotransmitters such as excitatory amino acids (EAAs) and substance P, inhibitory neurotransmitters such as opioids, adenosine, and γ-aminobutyric acid (GABA), and their combination (Dickenson *et al.*, 1997). MDH neurons, compared with DH neurons, have not been much studied with patch-clamp techniques (Chen *et al.*, 1995a, b).

This study was performed to establish a method for isolating and preparing MDH neurons for patch-clamp study and microfluorometry. We, also, identified voltage-sensitive $Ca^{2+}$ channels (VSCC) using patch-clamp and fura-2-based microfluorometry because VSCC plays an important role in neurotransmitter release in the central nervous system, and identification of VSCC is necessary to understand the pain modulation mechanism mediated by VSCC in MDH.

## Materials and Methods

### *Isolation and preparation of medullary dorsal horn (MDH) neurons*

We modified the spinal DH preparation previously described (Rusin *et al.*, 1992) in order to obtain single isolated MDH neurons. Sprague-Dawley rats, 1 to 2 weeks old, were decapitated, and the lower medulla was quickly removed and put into a cold oxygenated (95% $O_2$ and 5% $CO_2$) incubation buffer solution (see below). The tissue was sliced transversly in 400 μm sections with a Vibratome and then incubated in an oxygenated buffer solution for 30 min. The slices were then sequentially treated with pronase (1 mg/5 ml, Sigma) and thermolysin (1 mg/5 ml, Sigma) at 32°C for 20 min, respectively. After incubation, slices were washed with enzyme-free incubation buffer solution and stored at room temperature. Immediately before an experiment, slices were transferred to a tissue culture dish (35 mm in diameter, Falcon 3801) containing dissecting solution, and the MDH region was dissected out using small hand-held needles. The tissue was then mechanically dissociated under a stereomicroscope using a series of fire-polished glass pipettes with variously sized orifices. Single isolated cells were allowed to settle to the bottom of the experimental chamber.

### *Microfluorometry*

The acetoxymethyl-ester form of fura-2 (Fura-2/AM; Sigma, USA) was used as the fluorescent $Ca^{2+}$ indicator. The isolated MDH cells were loaded with fura-2/AM (2 μM) for 30 min at room temperature, after the cells were placed on a coverslip. Cells were then rinsed and visualized using an epifluorescence microscope (Axiovert 100, Zeiss) with an oil immersion objective lense at X400 magnification. The chosen cells were illuminated with a 75 W xenon arc lamp that passed light through a dual spectrophotometer (Photon Technology International; PTI, USA). The excitation wavelengths (340 and 380 nm) were altered by a spinning chopper (60 Hz), and the emitted light, selected at 510 nm, was collected by a photomultiplier. Data were analyzed using Felix software (PTI, USA).

### *Current recordings*

A whole cell patch-clamp technique (Hamill *et al.*, 1981) was used to record macroscopic currents of MDH neurons. Coverslips with MDH neurons were transferred to a recording chamber(containing external solution) which was mounted onto an inverted microscope (Olympus IMT-2, Japan). Electrodes were made from microcapillary glass tube (Chase instruments Co., USA) by using a series of two pulls on a vertical microelectrode puller (PP-83, Narishige, Japan), tip resistance was 2 to 5 M . Electrodes were placed onto the cell membrane with a micromanipulator (Burleigh, USA). A gigaseal was formed spontaneously or by slight negative suction. Conventional whole cell methods were used to record both $Na^+$ and $Ca^{2+}$ currents. After allowing the cells to equilibrate for at least 5 min, all experiments were performed. Whole-cell ionic currents (I) were measured with an Axopatch 1C amplifier (Axon Instruments, USA) at room temperature (18 to 25°C). Application of command potential and data acquisition were performed on-line by means of an IBM-compatible 486 computer. Currents were filtered at 5 KHz, digitally sampled via an analogue-to-digital

converter (TL-1 DMA interface, Axon Instruments, USA) and analyzed with pClamp 6 software (Axon Instrument).

### *Experimental solutions*

Incubation buffer solution was composed of (in mM) NaCl, 124; KCl, 5; $KH_2PO_4$, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 24; and glucose, 10. The proteases (pronase and themolysin) were added to the incubation buffer solution for enzyme treatment. The dissection solution was composed of (in mM) NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$ ,2; HEPES, 10; and glucose, 10; with a final pH of 7.4 (adjusted with 1 M tris aminomethane base). The external and internal solutions used for electrophysiological recordings were designed to record calcium-channel currents and minimize other ion-channel currents. Pipette solution was composed of (in mM) CsCl, 120; TEA-Cl, 20; $CaCl_2$, 1; $MgCl_2$, 2; Mg-ATP, 2; EGTA, 11; HEPES, 20; with a final pH of 7.3 (adjusted with CsOH). External solution was composed of (in mM) NaCl, 130; $CaCl_2$, 10; KCl, 5; glucose, 5; HEPES, 10; with a final pH of 7.3 (adjusted with NaOH). To identify voltage-sensitive $Ca^{2+}$ channels using fura-2-based microfluorometry, high $K^+$ saline solution (50 mM $K^+$) was made by replacing an equivalent amount of NaCl in normal saline solution with KCl. Normal saline solution (in mM) was NaCl, 120; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 5; HEPES, 5; and glucose, 10 (pH 7.4). Calcium-free saline solution was prepared by adding 1 mM EGTA and omitting $CaCl_2$ to each saline solution.

## Results

### *Acute isolation of MDH neurons*

MDH neurons were acutely isolated from lower brainstem slices cut 2 to 3 mm to caudal to the level of obex. MDH neurons prepared according to

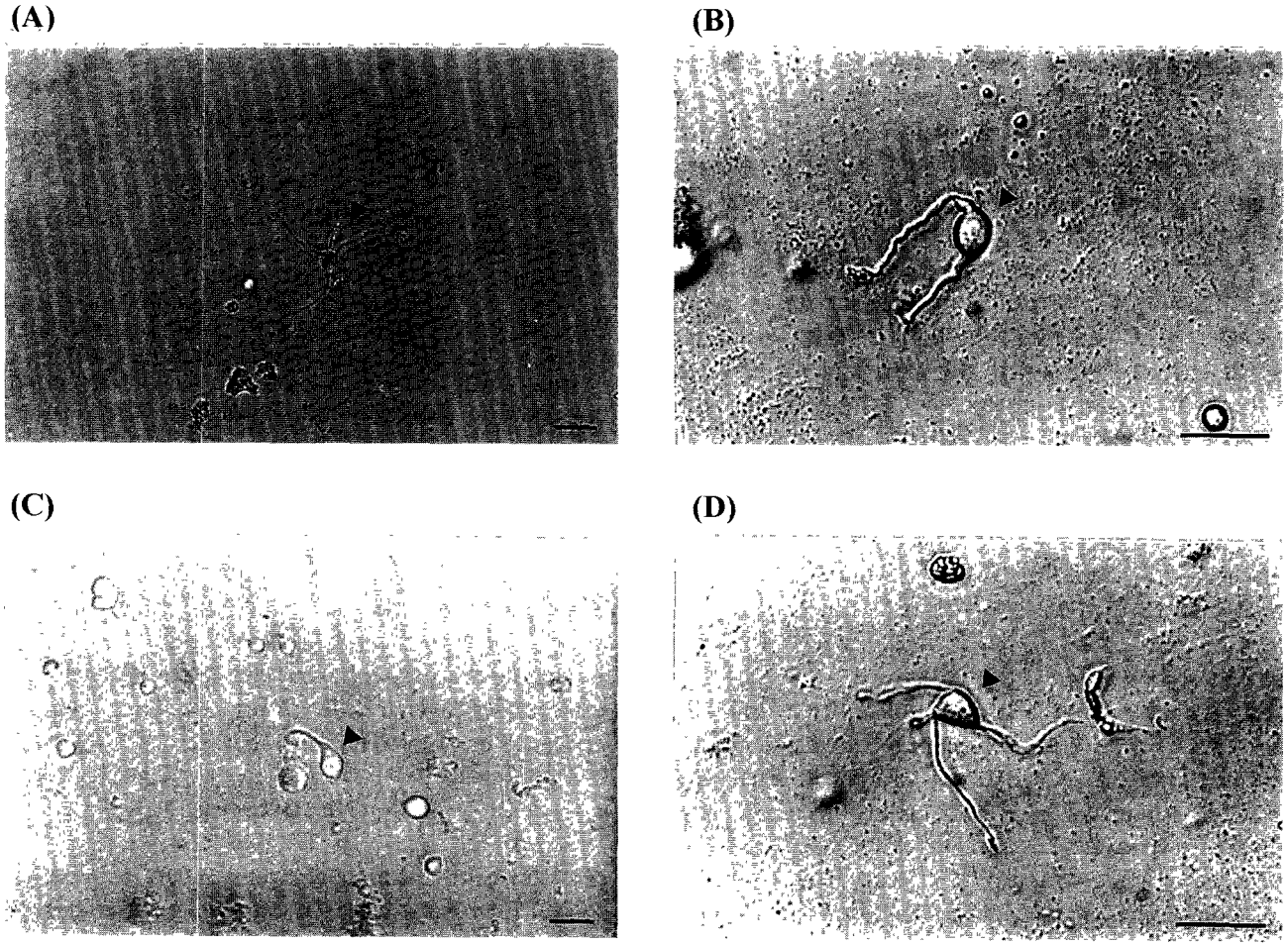


**Fig. 1.** Microscopic feature of representative isolated MDH neurons. Multipolar (A), bipolar (B), unipolar (C) and pyramidal cells (D) were obtained. Scale bar=30 μm.

the protocol described in materials and methods contain various cellular types with respect to their morphological characteristics. Fig. 1 shows microscopic features of representative isolated MDH neurons. Multipolar (A), bipolar (B), unipolar (C) and pyramidal cells (D) were observed. Spherical or oval shaped cells with their diameters ranging from 15 to 30 μm, were also obtained. The experiments were only performed in the healthy neuronal cells, which were highly refractile under a phase-contrast microscope.

### *Microfluorometry*

After depolarizing the cell with a high $K^+$ saline bath solution, $[Ca^{2+}]_i$ was measured using fura-2-based microfluorometry. Fig. 2A shows that depolarization induced by 30 sec exposure to 50 mM $K^+$ increased $[Ca^{2+}]_i$ from 2.1 ($Ca^{2+}$ ratio; 340 nm/380 nm excitation wave length) to 3.6 (increase of $[Ca^{2+}]_i$ was observed in the other 9 cells). The increase of $[Ca^{2+}]_i$ was reproducible when the cell was sequentially exposed to 50 mM $K^+$ three times at intervals of 3 min, but the peak level of $[Ca^{2+}]_i$ had a tendency to be reduced. To identify whether the increases of $[Ca^{2+}]_i$ were caused by calcium influx from the extracellular bath solution or release from intracellular calcium stores, we observed the changes of $[Ca^{2+}]_i$ using Ca $^{2+}$ free (CF) high $K^+$ saline bath solution. Exposure to CF high $K^+$ bath solution for 20 sec just after normal bath (NB) solution evoked a smaller peak than the peak induced by high $K^+$ bath solution (Fig. 2B). However, when the cell was exposed to CF high $K^+$ bath solution for 20 sec after the perfusion of CF normal bath (NB) solution to eliminate all the residual calcium in the bath, depolarization did not cause any increase in $[Ca^{2+}]_i$. In the same cell, the application of high $K^+$ saline bath solution containing $Ca^{2+}$ caused again the increase of $[Ca^{2+}]_i$ (Fig. 2C).

### *Current recordings*

$Ca^{2+}$ currents were recorded with the whole-cell patch-clamp technique under experimental conditions designed to record both voltage-sensitive $Na^+$ and $Ca^{2+}$ currents. In a bath solution of 10 mM $Ca^{2+}$ and 140 mM $Na^+$, almost every MDH neuron expressed voltage-sensitive $Ca^{2+}$ and $Na^+$ currents in response to a depolarizing test potential. Fig. 3A illustrates representative $Na^+$ and $Ca^{2+}$ current traces evoked during various depolarizations, from a holding potential of −80 mV. Both $Na^+$ currents, which were rapidly activated and inactivated, and slowly activated and long lasting $Ca^{2+}$ currents were shown in each current trace recorded. The current-voltage relationship of the peak $Na^+$ currents are plotted as in Fig. 3B.

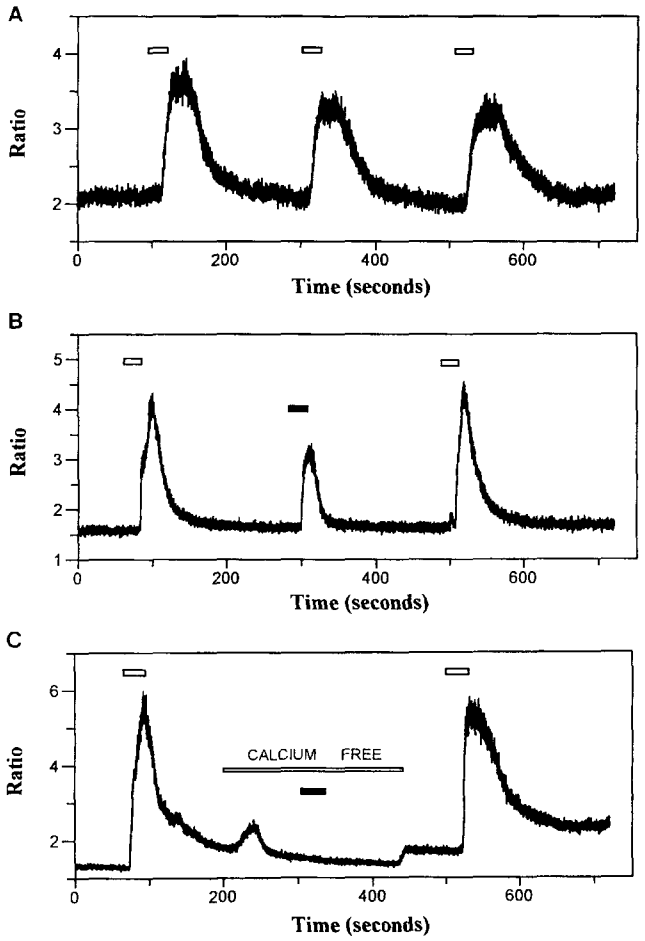


**Fig. 2.** Responses of MDH neurons to membrane depolarization induced by exposing them to high $K^+$ (50 mM) bath solution with (▭)or without (▬) of $Ca^{2+}$. A. The increase of $[Ca^{2+}]_i$ evoked by the superfusion of high $K^+$ bath solution containing $Ca^{2+}$ was reproducible. B. Exposure to high $K^+$ bath solution with no $Ca^{2+}$ just after normal bath evoked a smaller peak (second peak) compared to those induced by the high $K^+$ bath solution with $Ca^{2+}$ (first and third peaks). C. Application of a high $K^+$ bath solution after superfusion of $Ca^{2+}$-free normal bath solution for at least 1.5 min caused no changes of $[Ca^{2+}]_i$.

## Discussion

### *Isolation and identification of MDH neurons*

MDH neurons isolated by the method described in this study produced a wide range of yields, from almost zero to numerous healthy neuronal cells.

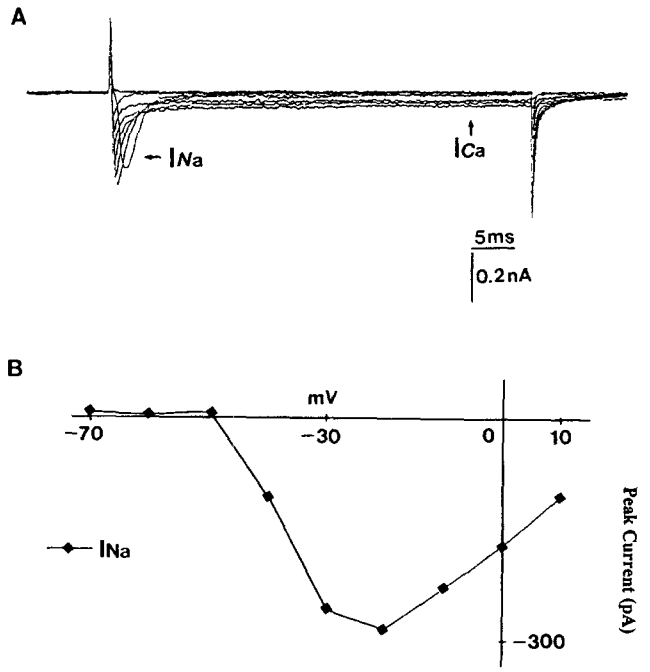


**Fig. 3.** A. Family of representative voltage-sensitive $Na^+$ and $Ca^{2+}$ currents evoked by depolarization to between −70 and +10 mV in 10 mV increments from a holding potential of −80 mV. B. Current-voltage relationship for the voltage-sensitive $Na^+$ acurrent at the peak influx in the cell depicted in A.

As discussed by Kay and Wong (1986), a number of factors can influence the yield: different saline solutions, mechanical regimes and enzymatic digestion. We believe that all these factors could affect the yield. According to this method, morphologically a distinct MDH neurons were isolated. Because the MDH is anatomically similar to the spinal dorsal horn (Dubner *et al.*, 1978), we tried to identify the cellular types isolated with reference to spinal dorsal horn cells. As demonstrated in Fig. 1, Multipolar (A), bipolar (B), unipolar (C) and pyramidal cells (D) were obtained. Six lamina, first described by Rexed (1952), make up the spinal dorsal horn. The neurons of lamina I have been classically divided into large marginal cells (so called marginal cells of Waldeyer), characterized by wide-ranging horizontal dendrites, and smaller neurons (Willis and Coggeshall, 1991). The multipolar cell (Fig. 1A) seems to be the marginal cell of lamina I. Likewise, there is the possibility that the bipolar (B) or unipolar (C) cells are the stalked cell or islet cells in lamina II (substantia gelatinosa). Pyramidal cells in lamina III that are flattened in the frontal plane have a similar morphology to the cell of Fig. 1D (Willis and Coggeshall, 1991). However, we cannot deny the fact that it is difficult to identify different cell types considering only their morphology once a tissue has been dissociated. In addition, dendrites and axons are usually severed during enzymatic and mechanical treatment, which can yield round or elliptical cells. However, the round and elliptical cells that were highly refractile under a phase-contrast microscope also showed good responses in this experiment.

***Identification of voltage-sensitive $Ca^{2+}$ channel***

VSCC, through which $Ca^{2+}$ influx has been shown to trigger neurotransmitter release from the nerve terminal in the central nervous system, is demonstrated here in MDH neurons with patch-clamp and fura-2-based microfluorometry techniques. Clearly, the regulation of this influx pathway represents a potential mechanism for the modulation of neurotransmission. Because orofacial pain transmission is largely modulated in the MDH area through peripheral afferent inputs and the descending CNS pathway (Sessle, 1989), identification of VSCC is a prerequisite for the understanding of neurotransmission modulation in MDH. First, an increase of $[Ca^{2+}]_i$ after $K^+$ application was observed using fura-2-based microfluorometry (Fig. 2A). This indicates that membrane depolarization induces an increase of $[Ca^{2+}]_i$ via $Ca^{2+}$ influx, $Ca^{2+}$ release from calcium reservoir sites or both. To identify the sources of increased intracellular $Ca^{2+}$, we tried to determine the effect of CF high $K^+$ bath solution, which can exclude $Ca^{2+}$ influx from extracellular bath solution, on the $[Ca^{2+}]_i$. When the CF high $K^+$ bath solution was applied just after the normal bath solution, a small peak, compared to the peak induced by high $K^+$ bath solution (Fig. 2B, first and third peaks), was evoked (Fig. 2B, second peak). This may be due to the residual $Ca^{2+}$ left after the change of bath solution. Therefore, in order to remove residual $Ca^{2+}$ in the recording chamber, we applied CF normal bath solution for at least 1.5 min before the application of CF high $K^+$ bath solution. As depicted in Fig. 2C, the application of CF high $K^+$ bath solution caused no change in $[Ca^{2+}]_i$, even though it induced membrane depolarization. Also, when high $K^+$ normal bath solution was applied again to the same cell, the $[Ca^{2+}]_i$ was elevated (Fig. 2C, second peak). These results suggest that there exist VSCC

in MDH neurons. We therefore tried to identify directly the existence of VSCC using electrophysiological methods. As expected from the spectrofluorometry experiment, the whole-cell patch clamp data demonstrate that the macroscopic inward $Ca^{2+}$ current in freshly isolated rat MDH cells is a voltage-sensitive $Ca^{2+}$ current ($I_{Ca}$). $I_{Ca}$, which was recorded with the Na+ current, was to be a high-voltage-activated $Ca^{2+}$ current on the basis of its biophysical properties (Fig. 3). The long lasting properties of $I_{Ca}$ were confirmed using the depolarizing command potential of 350 msec duration (Data not shown). The current is similar to the $I_{Ca}$ previously described in spinal dorsal horn neurons, which was studied in transverse slices (Ryu and Randic, 1990), and in the labelled trigeminothalamic cells (Huang, 1989). In addition, the voltage-sensitive $Na^+$ current was also recorded, which has different biophysical kinetics, such as rapid activation and inactivation compared to $I_{Ca}$ (Fig. 3).

These facts indicate that electrophysiological activities are well preserved in the acutely isolated neurons, and these cells can be effectively used for biophysical and pharmacological studies, including those aimed at understanding the pain modulation mechanism mediated by VSCC in MDH.

## Acknowledgements

This paper was supported by the research fund (L-42) for basic Medicine (1996) from the Ministry of Education of Korea. The authors would like to thank Jehoon Lee for his assistance in the revision process of the manuscript.

## References

Cerne, R., Rusin, K.I. and Randic, M.: Enhancement of the N-methyl-D-aspartate response in spinal dorsal horn neurons by cAMP-dependent protein kinase. Neurosci. Lett., 161: 124-128, 1993.

Chen, L., Gu, Y. and Huang, L.-Y.M.: The opioid peptide dynorphin directly blocks NMDA receptor channels in the rat. J. Physiol., 482.3: 575-581, 1995a.

Chen, L., Gu, Y. and Huang, L.-Y.M.: The mechanism of action for the block of NMDA receptor channels by the opioid peptide dynorphin. J. Neurosci., 15: 4602-4611, 1995b.

Dickenson, A.H., Chapman, V. and Green, G.M.: The Pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. Gen. Pharm., 28: 633-638, 1997.

Dubner, R., Sessle, B.J. and Storey, A.T.: The neural basis of oral and facial function. New York, Plenum Press, 1978.

Hamill, 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. Eur. J. Physiol., 391: 85-100, 1989

Hu, J.W., Dostrovsky, J.O. and Sessle, B.J.: Functional properties of neurons in cat trigeminal subnucleus caudalis(medullary dorsal horn). I. Responses to oral-facial noxious and nonnoxious stimuli and projections to thalamus and subnucleus oralis. J. Neurophysiol., 45(2): 173-192, 1981.

Huang, L.-Y.M.: Calcium channels in isolated rat dorsal horn neurones, including labelled spinothalamic and trigeminothalamic cells. J. Physiol., 411: 161-177, 1989.

Kay, A.R. and Wong, R.K.S.: Isolation of neurons suitable for patch-clamping from adult mammalian central nervous systems. J. Neurosci., M. 16: 227-238, 1986.

Kolaj, M., Cerne, R., Randic, M.: The opioid peptide dynorphin modulates AMPA and kainate responses in acutely isolated neurons from the dorsal horn. Brain Res., 671: 227-244, 1995.

Randic, M., Hecimovic. H. and Ryu, P.D.: Substance P modulates glutamate-induced currents in acutely isolated rat spinal dorsal horn neurones. Neurosci. Lett., 117: 74-80,1990.

Rusin, K.I. and Randic, M.: Modulation of NMDA-induced currents by μ-opioid receptor agonist DAGO in acutely isolated rat spinal dorsal horn neurons. Neurosci. Lett., 124: 208-212, 1991

Rusin, K.I., Bleakman, D., Chard, P.S., Randic, M. and Miller, R.J.: Tachykinins potentiate N-methyl-D-aspartate responses in acutely isolated neurons from the dorsal horn. J. Neurochem., 60(3): 952-960, 1993.

Rusin, K.I., Ryu, P.D. and Randic, M.: Modulation of excitatory amino acid responses in rat dorsal horn neurons by tachykinins. J. Neurophysiol., 68: 265-286, 1992.

Ryu, P.D. and Randic, M.: Low- and high-voltage-activated calcium currents in rat spinal dorsal horn neurons. J. Neurophysiol. 63(2): 273-285, 1990.

Sah, D-W.Y.: Neurotransmitter modulation of calcium current in rat spinal cord neurons. J. Neurosci., 10: 136-141, 1990.

Sessle, B.J.: The neurobiology of facial and dental pain:

present knowledge, future directions. J. Dent. Res., 66: 962-981, 1987.

Sessle, B.J.: Neural mechanisms of oral and facial pain. Otolaryngologic Clinics North Am., 22(6): 1059-1072, 1989.

Sessle, B.J., Hu, J.W., Dubner, R. and Lucier, G.E.: Functional properties of neurons in cat trigeminal subnucleus caudalis (medullary dorsal horn). II. Modulation of responses to noxious and nonnoxious stimuli by periaqueductal gray, nucleus raphe magnus, cerebral cortex, and afferent influences, and effect of naloxone. J. Neurophysiol., 45(2): 193-207, 1981.

Wang, R.A., Randic, M.: Activation of μ-opioid receptor modulated GABAA receptor-mediated currents in isolated spinal dorsal horn neurons. Neurosci. Lett., 180: 109-113, 1994.

Willis, W.D. and Coggeshall, R.E.: Sensory mechanism of spinal cord. 2nd. ed. pp 79-151, Plenum Press. New York and London, 1991.