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L-Glutamate, NMDA and Substance P Increase Intracellular Calcium Concentration in Medullary Dorsal Horn Neurons: Investigation of Trigeminal Pain Mechanism I

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Medually dorsal horn (MDH) neurons, known as the sensory neurons, are involved in orofacial pain transmission. In the present study, we investigated the types of neurotransmitters that affect intracellular free calcium concentration ($[Ca^{2+}]_i$), and which modulate pain sensation in the trigeminal sensory pathway. $[Ca^{2+}]_i$ was measured in fura-2 acetoxymethyl ester (fura-2) loaded MDH neurons in neonatal rat pups by microspectrofluorimetry. Most of the neurons(91.7%) responded to L-glutamate by increasing $[Ca^{2+}]_i$ and this response was reversible and consistent. $[Ca^{2+}]_i$ increase was dose dependent, i.e., 0.24±0.06 ($\Delta F_{340}/F_{380}$ ratio, n=2), 1.77±0.35 (n=10) and 1.82±0.15 (n=2), at 30 μM, 300 μM and 1 mM of glutamate, respectively. Substance P(SP, 1 nM) also evoked a $[Ca^{2+}]_i$ increase (1.6±0.62, n=2) in MDH neurons; however, this occurred in only 18.2% of neurons tested. In contrast to glutamate, the SP receptor showed a rapid desensitization. N-methyl-D-aspartate (NMDA) also increased $[Ca^{2+}]_i$ in 21.1% of the neurons tested. NMDA-induced $[Ca^{2+}]_i$ increase was not observed in the absence of extracellular Ca^{2+}, suggesting that NMDA-induced $[Ca^{2+}]_i$ increase is due to an influx of extracellular Ca^{2+}. The coexpression of the SP and NMDA receptors with the glutamate receptor or the SP receptor with the NMDA receptor was observed in these neurons, although both expression percentages were low. In conclusion, L-glutamate, SP and NMDA functional receptors are expressed early in neonatal MDH neurons and may act as excitatory modulators in MDH neurons by increasing $[Ca^{2+}]_i$.

Key words : medullary dorsal horn, L-glutamate, NMDA, substance P, intracellular free calcium

Introduction

The medullary dorsal horn (MDH), subnucleus caudalis of the trigeminal spinal tract nucleus, is a major relay site for orofacial pain transmission (Sessle, 1989). It contains many putative neurotransmitters, including excitatory and inhibitory amino acids and peptides (Sessle, 1987). Its anatomy and function are similar to that of the spinal dorsal horn (Dubner *et al.*, 1978). MDH neurons receive noxious input from the primary afferent fibers, integrate modulatory influences from descending and local circuits and forward the information to the thalamus and somatosensory cortex (Willis and Coggeshall, 1991). However, the mechanism of sensory modulation in the MDH is remains unclear.

The use of acutely isolated DH neurons permitted the analysis of the direct postsynaptic interactions between excitatory neurotransmitters, e.g., excitatory amino acid (EAA) and substance P, and inhibitory neurotransmitters, e.g., opioid, adenosine and GABA (Dickenson *et al.*, 1997). Even though the responses of MDH neurons have been widely investigated in *in vivo* preparations (Hu *et al.*, 1981; Sessle *et al.*, 1981), they have not been comprehensively compared with DH neurons, in *in vitro* preparations (Chen *et al.*, 1995a, b).

Recently, some of the receptor agonists have been shown to elevate $[Ca^{2+}]_i$ in the dorsal root and the trigeminal ganglion neurons of neonatal rats (Bowie *et al.*, 1994; Park *et al.*, 1997). However, no attempts have been made as yet to study the effects of various neurotransmitters on $[Ca^{2+}]_i$ in MDH neurons. Because of the importance of these neurons in orofacial pain transmission, the modulation of the activities of these neurons might prove of interest. The aim of this experiment was to determine the kinds of neurotransmitters that elevate $[Ca^{2+}]_i$, which plays a role in neurotransmitter release. For this purpose, three agonists, L-glutamate, N-methyl-

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D-aspartate (NMDA) and substance P (SP), were investigated for their ability to increase $[Ca^{2+}]_i$ from fura-2 acetoxymethyl ester (fura-2) loaded MDH neurons by microspectrofluorometry.

Materials and Methods

Isolation and preparation of medullary dorsal horn (MDH) neurons

Isolation and preparation of MDH neurons was performed according to a previously described method (Kay and Wong, 1986; Oh *et al.*, 1998). In order to obtain single isolated MDH neurons, one to two-week-old Sprague-Dawley rats were decapitated, and the lower medulla was quickly removed and place in cold, oxygenated (95% O_2 and 5% CO_2) incubation buffer solution (see below). The tissue was transversely sliced in 400 m sections with a Vibratome and then incubated in oxygenated buffer solution for 30 min. The slices were sequentially treated in solution with pronase (1 mg/5 ml) and thermolysin (1 mg/5 ml) at 32 for 20 min. Pronase and thermolysin were purchased from Sigma (St. Louis, MO, USA). After incubation, the slices were washed with enzyme free incubation buffer solution and stored at room temperature. Immediately before an experiment, the 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 various orifice sizes. Single isolated cells were allowed to settle to the bottom of the experimental chamber.

Fluorescent dye loading and $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was determined using a microscope photometry technique with a Ca^{+2} sensitive dye, fura-2 acetoxymethyl ester (fura-2). Isolated MDH neurons were loaded with 2 μM fura-2 for 30 min at room temperature. After washing the neurons three times in a standard bath solution, the MDH neurons were kept on ice until use. After MDH neurons had adhered to the bottom of the tissue bath precoated with Cell-Tak, they were visualized using an epifluorescence microscope (Axiovert 10, Zeiss, Germany). The fluorescent probe fura-2 was excited using a collimated beam of light from a 75 W Xenon arc lamp and passed through a dual spectrophotometer (Photon Technology International, Lawrenceville, NJ, U.S.A) that altered wavelengths from 340 to 380 nm using a chopper (60 Hz). The emitted light, selected at 510 nm, was collected by a photomultiplier. The flow rate was 1 ml/min and the cell chamber capacity was 300 μl. All agonists were added to the standard bath solution. The Ca^{+2} fluorosence ratio was recorded and analyzed using Felix software (PTI, U.S.A).

Experimental solutions

Incubation buffer solution was composed of 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 24 mM $NaHCO_3$ and 10 mM glucose. Two proteases (pronase and themolysin) were added to the incubation buffer solution for enzyme treatment. The dissection solution was composed of 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose with a final pH of 7.4 (adjusted with 1 M Tris aminomethane base). Standard bath solution was composed of 130 mM NaCl, 10 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 10 mM HEPES with a final pH of 7.4 (adjusted with NaOH). To identify voltage-sensitive Ca^{2+} channels by fura-2-based microfluorometry, high K^+ saline solution (50 mM K^+) was made by replacing an equivalent amount of NaCl in standard bath solution with KCl. Ca^{2+} free bath solution was prepared by adding 1 mM of EGTA and omitting $CaCl_2$ from the standard bath solution. L-glutamate, SP and NMDA were purchased from Sigma. Dispase (grade II) was purchased from Boehringer Manheim (Manheim, Germany). Fura-2 was purchased from Molecular probe (Eugene, OR, USA).

Results

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) increase evoked by depolarization

Figure 1 shows a transient increase of $[Ca^{2+}]_i$ evoked by high K^+ bath solution (50 mM KCl) in MDH neuron for 10 sec (indicated by short dark bars). KCl was replaced with an equimolar amount of NaCl in the standard bath solution. Depolarization induced $[Ca^{2+}]_i$ increase was observed in all neurons tested (n=6). $[Ca^{2+}]_i$ increased from 1.4±0.05 (Ca^{2+} fluoresence ratio F_{340}/F_{380}, Mean±SE) to 2.58±0.16 (n=6). In the absence of Ca^{2+}, high K^+ (indicated by white bars) did not evoke an increase in $[Ca^{2+}]_i$, suggesting that $[Ca^{2+}]_i$ increases induced by high K^+ solution is due to Ca^{2+} influx via voltage-dependent Ca^{2+} channels.

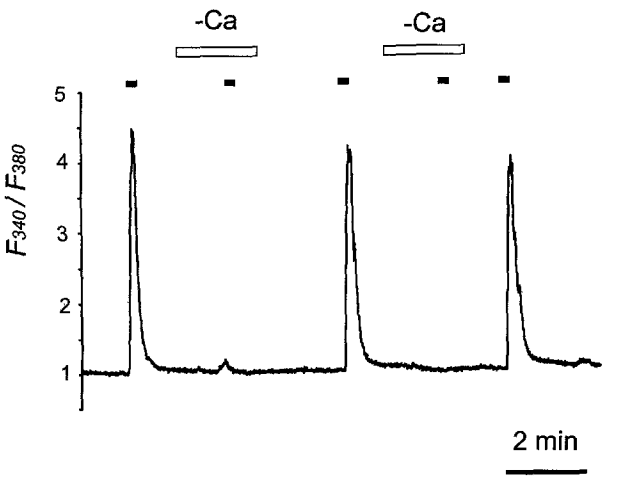

Fig. 1. Depolarization-induced $[Ca^{2+}]_i$ increase in MDH neurons. 50 mM KCl replaced an equimolar amount of NaCl in the bath solution (indicated by a dark bar). An $[Ca^{2+}]_i$ increase was not observed in the Ca^{2+} free bath solution.

Glutamate increases $[Ca^{2+}]_i$

Figure 2A shows a typical $[Ca^{2+}]_i$ response to glutamate at concentrations of 30 μM, 300 μM and 1 mM. 30 μM glutamate produced a slight Ca^{2+} transient increase from 1.15 in the resting state to 1.45. After $[Ca^{2+}]_i$ recovered to the prestimulus level, the MDH neuron was stimulated again with 300 μM glutamate (middle trace). The magnitude of the $[Ca^{2+}]_i$ increase of 2.1 was much higher than that induced by 30 μM glutamate. Finally, 1 mM glutamate increased $[Ca^{2+}]_i$ to 2.2, which was not very significantly different from that achieved by 300 μM glutamate. The $[Ca^{2+}]_i$ increase induced by 30 μM, 300 μM and 1 mM glutamate were 0.24±0.06 (n=2), 1.77±0.35 (n=10) and 1.82±0.15 (n=2), respectively. Figure 2B shows that the effect of 300 μM glutamate on $[Ca^{2+}]_i$ is reversible and consistent. The magnitude of the $[Ca^{2+}]_i$ transient increase was not significantly decreased by repeated stimulations. The maximum $[Ca^{2+}]_i$ induced was 2.75 from an initial resting value of 1.2. We also stimulated the MDH neuron with 10 μM of NMDA before glutamate stimulation, however this did not evoke any $[Ca^{2+}]_i$ increase (indicated by N on the first dark bar).

SP increases $[Ca^{2+}]_i$

Figure 3A shows the response of an MDH neuron to 1 nM SP and 300 μM glutamate. Threshold

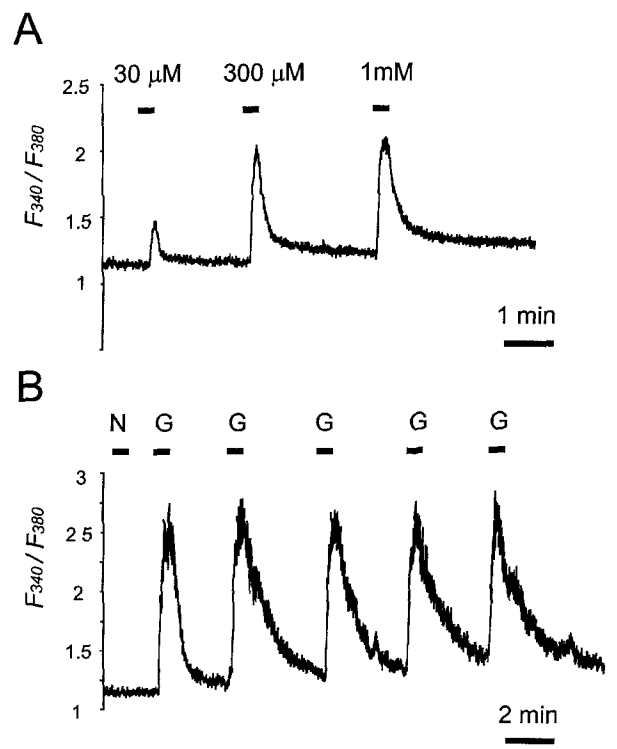

Fig. 2. (A) Dose-dependent $[Ca^{2+}]_i$ increase by 30 μM, 300 μM and 1 mM of glutamate(indicated by G on the dark bar). (B) Consistent and reversible increase of $[Ca^{2+}]_i$ induced by repeated stimulation with 300 μM glutamate. N, 10 μM NMDA.

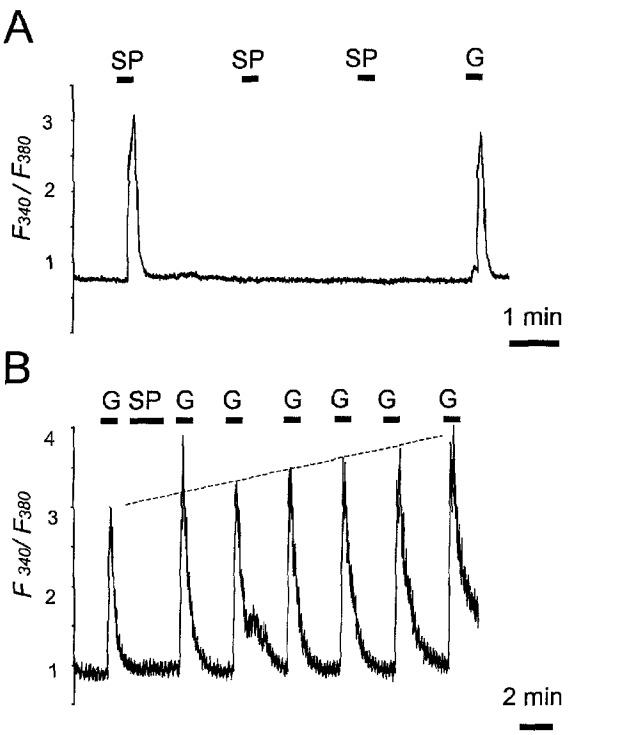

Fig. 3. (A) $[Ca^{2+}]_i$ increase evoked by 1 nM SP. The second and the third application of SP did not increase $[Ca^{2+}]_i$. The second $[Ca^{2+}]_i$ transient increase was induced by 300 μM of glutamate (G). B) Preincubation with SP potentiates a glutamate-induced $[Ca^{2+}]_i$ increase.

concentration of SP were estimated to lie between 0.1 nM and 1 nM, since 0.1 nM SP did not evoke a $[Ca^{2+}]_i$ increase (data not shown), but 1 nM SP evoked a clear $[Ca^{2+}]_i$ increase from 1.35±0.1(n=2) in resting state to 2.95±0.52 (n=2). In contrast to glutamate, the SP receptor showed a rapid desensitization. After the first $[Ca^{2+}]_i$ transient increase evoked by 1 nM SP, subsequent applications of SP did not cause a $[Ca^{2+}]_i$ increase. However, subsequent stimulation with 300 μM glutamate showed a substantial increase in $[Ca^{2+}]_i$. The coexpression of the SP receptor with the glutamate receptor was observed in only one neuron in a total five experiments (20%). Fig. 3B shows a representative figure of $[Ca^{2+}]_i$ transient increases induced by repeated stimulation with 300 μM of glutamate in an MDH neuron which was preincubated with SP for 2 mins. SP preincubation potentiated a glutamate-induced $[Ca^{2+}]_i$ increase (n=2).

NMDA increases $[Ca^{2+}]_i$

Figure 4A shows functional coexpression of NMDA and glutamate receptors in an MDH neuron. There was no $[Ca^{2+}]_i$ response to 1 nM SP in this neuron (indicated by SP on the third bar). The $[Ca^{2+}]_i$ response evoked by 100 M of NMDA decreased

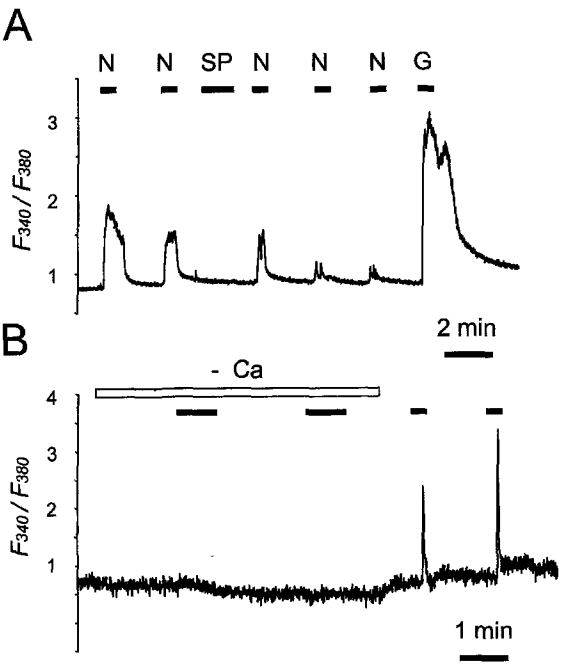

Fig. 4. (A) $[Ca^{2+}]_i$ increase evoked by 100 μM NMDA. $[Ca^{2+}]_i$ responses were decreased following repeated stimulation. The same neuron responsed to 300 μM glutamate with a large increase of $[Ca^{2+}]_i$ (indicated by G on the bar) B) NMDA does not increase $[Ca^{2+}]_i$ in Ca^{2+} free bath solution, but does increase $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}.

following repeated stimulations, however this increase was not as prominent as that of the SP receptors. The coexpression of the NMDA receptor with the glutamate receptor was observed in only one neuron (25%) in a total of four experiments. Figure 4B shows the response of an MDH neuron to 10 μM of NMDA in Ca^{2+} free extracellular medium. NMDA did not evoke any $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+}. However, when Ca^{2+} is added to the extracellular medium, NMDA increased $[Ca^{2+}]_i$ in a dose dependent manner (the first peak at 10 μM and the second peak at 100 μM NMDA, respectively). The result suggests that the NMDA induced increase of $[Ca^{2+}]_i$ is due to Ca^{2+} influx from the extracellular medium.

Dose response curves and functional percentage expression of $[Ca^{2+}]_i$ by glutamate, SP and NMDA receptor

Figure 5A shows dose response curves for glutamate, SP and NMDA. The magnitude of $[Ca^{2+}]_i$ increase was: 0 (n=2) and 1.6±0.62 (n=2) at 0.1 nM and 1 nM of SP; 0.3±0.21 (n=2) and 1.0±0.19 (n=5) at 10 μM and 100 μM of NMDA; and 0.24±0.06 (n=2), 1.77±0.35 (n=10) and 1.82±0.15 (n=2) at 30 μM, 300 μM and 1 mM glutamate, respectively. The maximal $[Ca^{2+}]_i$ increase induced by 1 mM of glutamate was not significantly different from that induced by 300 μM of glutamate. Figure 5B shows a histogram of the functional expression percentage of each receptor. Neurons which respond to at least one of the three agonists (glutamate, SP or NMDA) were included in the data. The glutamate receptor was functionally expressed in most of the MDH neurons. Glutamate (300 μM) induced an $[Ca^{2+}]_i$ increase in 11 of 12 neurons (91.7%). In contrast, the functional expression percentages of NMDA and SP were relatively low. A $[Ca^{2+}]_i$ transient increase induced by 100 μM of NMDA was observed in four of 19 neurons tested (21.1%, Fig. 4B), whereas SP-induced $[Ca^{2+}]_i$ increase was observed in 2 of 12 neurons (18.2%). The coexpression percentages of the NMDA or SP receptor with the glutamate receptor, or of the NMDA receptor with the SP receptor were 25%, 20% and 50%, respectively.

Discussion

Intracellular free calcium concentration is strictly regulated in many excitable neurons, including cultured cerebellar neurons (Brorson *et al.*, 1991),

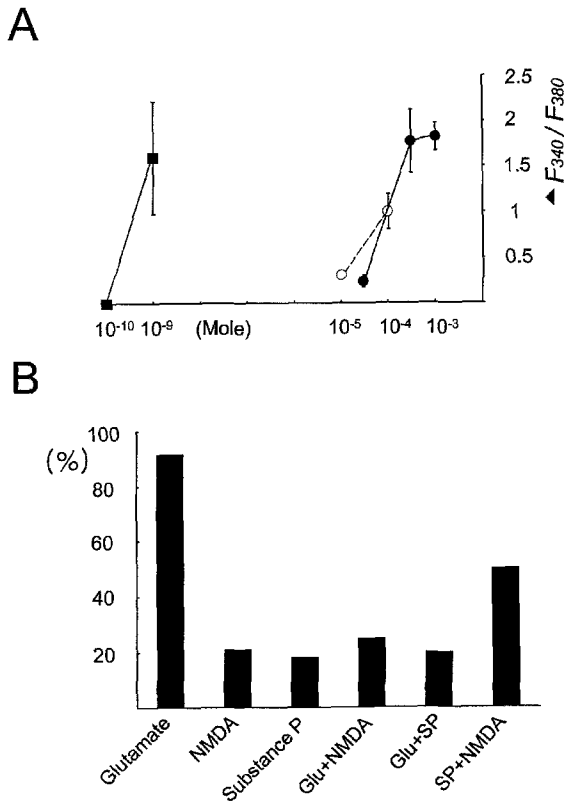

Fig. 5. A) Dose-response curves of glutamate(●), NMDA(○) and SP(■) at various concentrations. The vertical axis shows the amount of $[Ca^{2+}]_i$ increase induced by each agonist. B) Functional expression percentages of each receptor, and its coexpression with other receptors.

sympathetic neurons in bull-frogs (Marrion and Adams, 1992), cultured spinal cord neurons in mice (Mayer *et al.*, 1987), and dorsal root ganglion neurons in rats (Thayer and Miller, 1990). The excitability of MDH neurons are also regulated by $[Ca^{2+}]_i$. In this experiment, we investigated the effects of excitatory neurotransmitters on $[Ca^{2+}]_i$ in MDH neurons.

The profile of the $[Ca^{2+}]_i$ response to each neurotransmitter was different. Glutamate increased $[Ca^{2+}]_i$ in a dose-dependent manner and the magnitude of the $[Ca^{2+}]_i$ response evoked by glutamate was not decreased by repeated stimulations, suggesting that the glutamate receptor is not desensitized. In our experiment, the glutamate receptor was expressed early in most of the MDH neurons we examined (91.7%). In rat dorsal horn neurons, stimulation with excitatory amino acids (EAA), including glutamate, kainate, quisqualate and NMDA, activated inward currents (Murase *et al.*, 1989), and the glutamate-evoked currents were modulated by SP. These results suggested that post-synaptic mechanisms of SP may contribute to the regulation of glutamate-mediated excitatory transmission in the rat dorsal horn (Randic *et al.*, 1990). In these neurons, various excitatory neurotransmitters, including glutamate, consistentely increased $[Ca^{2+}]_i$ (Rusin *et al.*, 1993). Our results are in partial agreement with those reported for rat dorsal horn neurons. The glutamate evoked $[Ca^{2+}]_i$ increase was consistent and reversible. Moreover, SP preincubation potentiated a glutamate-induced $[Ca^{2+}]_i$ increase in this experiment. It is likely that the stimulation parameters required to release of SP would have a higher threshold (Verhage *et al.*, 1991). Therefore, with high rates of stimulation, the co-release of the two receptors greatly potentiates the postsynaptic actions of glutamate.

In contrast to the glutamate receptors, only a subpopulation of MDH neurons (21.1%) responded to NMDA by increasing $[Ca^{2+}]_i$. In the previous study, desensitization by NMDA was not observed in dorsal horn neurons, however in our studies, the NMDA-induced $[Ca^{2+}]_i$ response was decreased by repeated stimulation. Moreover, the $[Ca^{2+}]_i$ produced by NMDA was not potentiated by SP. In rat dorsal horn neurons, the increase in $[Ca^{2+}]_i$ produced by NMDA was potentiated by SP in eight of 17 cells (Rusin *et al.*, 1993). It is unlikely that the result of our experiment is due to the lack of response of cells to SP alone, since SP-induced potentiation was observed even in those neurons in which there was no change in $[Ca^{2+}]_i$ produced by SP (Rusin *et al.*, 1993). It is unclear at the moment why these differences exist. The expression percentage of SP seems to be much lower in MDH neurons (18.2% in this experiment) than that of dorsal horn neurons (35%), suggesting that the contribution of SP to orofacial sensory transmission is relatively less than the sensory transmission via dorsal horn neurons. The observed increase in $[Ca^{2+}]_i$ produced by NMDA in our experiment appeared to be dependent on the presence of extracellular Ca^{2+}, since NMDA-induced $[Ca^{2+}]_i$ increase was not observed in Ca^{2+} free bath solution. The existence of a voltage sensitive Ca^{2+} channel was confirmed in the MDH neurons (see Fig. 1).

However, the source of $[Ca^{2+}]_i$ evoked by SP remains controversial. Womack *et al.* (1988) reported that the increase in $[Ca^{2+}]_i$ produced by SP was the results of Ca^{2+} mobilization from an intracellular store. In contrast, Rusin *et al.*(1993) suggested that $[Ca^{2+}]_i$ produced by SP appeared to be due to Ca^{2+} influx from the extracellular medium, since such a

$[Ca^{2+}]_i$ increase was blocked by Ca^{2+} channel antagonist. In our present study, SP showed a marked desensitization compared with glutamate or NMDA. Such a rapid desensitization induced by SP (10^{-10} to 10^{-6} M) was also reported in rat dorsal horn neurons. SP-evoked currents reached a peak within 2-15 sec and desensitized in the continued presence of SP (Randic *et al.*, 1990). Although we did not investigate the source of intracellular Ca^{2+}, previous results(Randic *et al.*, 1990) suggest that the increase level of $[Ca^{2+}]_i$ produced by SP in this experiment seems to be due to Ca^{2+} influx from the extracellular medium.

In the coexpression study of these receptors, the coexpression percentage of NMDA or SP with glutamate was observed in a very low percentage of the neurons, i.e., 25% and 20%, respectively. Although the coexpression percentage of the NMDA receptor with the SP receptor (50%) in this experiment, corresponds to that in dorsal horn neurons (47%), we cannot rule out the possiblity of a rather high estimation due to the low expression percentage of each receptor in MDH neurons. In summary, receptors for glutamate, SP and NMDA are expressed in neonatal MDH neurons and all these receptors are involved in elevating $[Ca^{2+}]_i$. However, only a subpopulation of MDH neurons responded to NMDA and SP, suggesting that their contribution to orofacial sensory transmission is less than the sensory transmission via the dorsal horn neurons. It is hoped that this result may be useful to those wishing to further study sensory transmission via the trigeminal pathway and other pathways involving the spinal cord.

Acknowledgments

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