Korean J Physiol Pharmacol
Vol 3: 35–45, February, 1999

Calcium Modulates Excitatory Amino Acid (EAA)- and Substance P-induced Rat Dorsal Horn Cell Responses

Hong Kee Shin, Sok Han Kang, In Duk Chung, and Kee Soon Kim

Department of Physiology, School of Medicine, Institute of Biomedical Sciences, Hanyang University, Seoul 133–791, Korea

Excitatory amino acid (EAA) and substance P (SP) have been known to be primary candidates for nociceptive neurotransmitter in the spinal cord, and calcium ions are implicated in processing of the sensory informations mediated by EAA and SP in the spinal cord. In this study, we examined how Ca^{2+} modified the responses of dorsal horn neurons to single or combined iontophoretical application of EAA and SP in the rat. All the LT cells tested responded to kainate, whereas about 55% of low threshold (LT) cells responded to iontophoretically applied NMDA. NMDA and kainate excited almost all wide dynamic range (WDR) cells. These NMDA- and kainate-induced WDR cell responses were augmented by iontophoretically applied EGTA, but suppressed by Ca^{2+}, Mn^{2+}, verapamil and ω-conotoxin GVTA, effect of verapamil being more prominent and well sustained. Ca^{2+} and Mn^{2+} antagonized the augmenting effect of EGTA. On the other hand, prolonged spinal application of EGTA suppressed the response of WDR cell to NMDA. SP had triple effects on the spontaneous activity as well as NMDA-induced responses of WDR cells: excitation, inhibition and no change. EGTA augmented, but Ca^{2+}, Mn^{2+} and verapamil suppressed the increase in the NMDA-induced responses and spontaneous activities of WDR cells following iontophoretical application of SP. These results suggest that in the spinal cord, sensory informations mediated by single or combined action of EAA and SP can be modified by the change in calcium ion concentration.

Key Words: Dorsal horn neuron, Excitatory amino acids, Substance P, Calcium channel antagonists, Iontophoresis

INTRODUCTION

Curtis et al (1959) and Konishi & Otsuka (1974) respectively reported that glutamate and substance P (SP) produced powerful excitation of spinal neurons. Thereafter, accumulating evidences have suggested that excitatory amino acid (EAA) and SP play a major role in a nociceptive transmission in the spinal cord. Both EAA and SP are contained in small diameter primary afferent fibers which terminate in laminae I and II in the dorsal horn (De Biasi & Rustioni, 1988) and high density of their binding sites has been demonstrated in the superficial laminae of dorsal horn where many nociceptive afferent fibers terminate (Marshall et al, 1996). EAA and SP are released into the dorsal horn upon various noxious stilmuli, but not upon innocuous stimuli (Duggan et al, 1988; Skilling et al, 1988; Schaible et al, 1990; Kangrga & Randić, 1991; Paleckova et al, 1992). In the immunocytochemical study of the rat dorsal horn and dorsal root ganglion (DRG) cell, glutamate was observed to be colocalized with SP in the same axonal boutons and DRG cells (Battaglia & Rustioni, 1988; De Biasi & Rustioni, 1988). SP has been known to increase the release of EAA from the primary afferent nerve terminal (Smullin et al, 1990). Dorsal rhizotomy and neonatal capsaicin treatment also reduce EAA and SP release from primary afferent fibers (Jessell et al, 1979; Kangrga et al, 1990). These experimental findings imply that SP and EAA are candidate neurotransmitters for nociceptive information which are released from capsaicin-sensitive high threshold primary afferent fibers.

Many neurons activated by iontophoretic administration of EAA or SP are localized mainly in laminae I and II of the spinal cord and receive noxious inputs from afferent nerves (Randić & Miletic, 1977; Schneider & Perl, 1985). SP induces slow prolonged depolarization in the dorsal horn neurons

Corresponding to: Hong Kee Shin, Department of Physiology, School of Medicine, Hanyang University, Seoul 133-791, Korea

while EAA excites dorsal horn neurons with rapid onset and offset (De Koninck et al, 1992). Intrathecal application of SP and EAA elicit behavioral responses such as vocalization, scratching and biting of hindlimb (Raigorodsky & Urca, 1990; Smullin et al, 1990). EAA and SP are also implicated in the induction and maintenance of tonic pain by subcutaneous injection of formalin, kaolin and carrageenan (Sluka & Westlund, 1993; Traub, 1996). On the other hand, NMDA and non-NMDA receptor antagonists such as MK-801, AP5 and CNQX suppress hyperalgesia and autotomy resulting from peripheral nerve injury (Seltzer et al, 1991; Mao et al, 1992).

In the dorsal horn neurons, SP and EAA are known to cause an increase in intracellular concentration of calcium ions ($[Ca^{2+}]_i$) (Murphy et al, 1987; Heath et al, 1994) and induce slow inward current with characteristics of Ca^{2+} current (Murase et al, 1986). Both SP- and NMDA-induced increases in $[Ca^{2+}]_i$ were supposed to result from the entry of Ca^{2+} into the cell from the extracellular fluid because Ca^{2+}-free condition abolished SP- and NMDA-induced change in $[Ca^{2+}]_i$ (Murphy et al, 1987; Heath et al, 1994). Lanthanum blocks the SP-induced Ca^{2+} entry while AP5, MK-801 and Mg^{2+}, but not La^{3+}, antagonize the NMDA-induced increase in $[Ca^{2+}]_i$ (Murphy et al, 1987; Heath et al, 1994).

Calcium ions are involved in the control of physiological functions, including enzyme activity, membrane permeability, neurotransmitter release, and synaptic transmission (Olivera, 1994). Calcium channel blockers are also effective for the treatment of exertional angina and essential hypertension (Fleckenstein et al, 1985), and have anticonvulsant effect and neuroprotective action against ischemic brain damage (Zapater et al, 1997; Zapater et al, 1998). One of the important action of Ca^{2+} is its involvement in the processing of the nociceptive informations in the spinal cord. Ca^{2+} channel blockers antagonize nociceptive responses with different origins. L-, N-, and P-type voltage-sensitive Ca^{2+} channel blockers inhibit the normal and sensitized responses of WDR cells to C-fiber and thermal stimulation without any effect on the response to A-fiber stimulation (Shin et al, 1997). L-type Ca^{2+} channel blockers and thapsigargin block thermal hyperalgesia induced by intrathecally administered SP (Henry et al, 1996). L-type (verapamil, diltiazem, nifedipine) and N-type (ω-conotoxin, SNX-111) Ca^{2+} channel blockers dose-dependently suppress tonic pain responses induced by acute inflammation, thermal hyperalgesia and mechanical allodynia resulting from peripheral nerve injury (Gürdal et al, 1992; Chaplan et al, 1994; Malmberg & Yaksh, 1995; Neugebauer et al, 1996). It was reported that a continuous infusion of SNX-111 for 7 days neither developed tolerance nor reduced antinociceptive action of the drug (Malmberg & Yaksh, 1995).

Putting all these informations described together, we can reach the conclusion that SP and EAA are candidate neurotransmitters for nociceptive primary afferent fibers in the spinal cord and SP- and NMDA-induced increase in $[Ca^{2+}]_i$ could play an important role in the processing of nociceptive responses. The present study was designed to investigate the effects of Ca^{2+} and Ca^{2+} channel blockers on the responses of spinal dorsal horn neurons to iontophoretical application of EAA and SP.

METHODS

Sixty four adult male rats (Sprague-Dawley, 300~450 gm) were anesthetized with urethane (1.2 gm/kg, i. p.). External jugular vein was cannulated with polyethylene tube (PE-60) through which pancuronium bromide (0.3 mg/kg/hr) was continuously infused to paralyze the musculature. The rats were artificially ventilated and end-tidal CO_2 was maintained between 3.5 and 4.5%. Core temperature was kept near 38°C by a homeothermic blanket system (Harvard Apparatus, USA). Lumbar enlargement of spinal cord between T13 and L3 was exposed by laminectomy and the common peroneal and tibial nerves were dissected from the surrounding tissues at popliteal fossa. The exposed tissues were covered with warm liquid paraffin to prevent drying. After finishing all these surgical procedures, the rats were placed in a stereotaxic apparatus.

Spinal dorsal neurons were activated by electrical stimulation of the exposed peripheral nerve and their activities were extracellulary recorded with the central barrel of seven-barreled microelectrode which contained a low-impedence carbon filament. Once the single unit activity of dorsal horn neuron was recorded, type of the neuron was classified into wide dynamic range (WDR) cells and low threshold (LT) cell according to the response pattern to mechanical stimuli applied to the receptive field. Outer barrels of the 7-barreled microelectrode were used to apply drugs by microiontophoresis. In this experiment, the following substances were applied to the spinal cord by microiontophoresis: glutamate (0.2 M), N-methyl-D-aspartate (NMDA, 0.05 M), kainate (0.02 M), Ca^{2+} (0.1 M), Mn^{2+} (0.2 M), EGTA (0.1 M), verapamil (0.03 M), and ω-conotoxin GVIA (0.1 mM). All

solutions were at pH 7.5~8.0. All drugs were purchased from Sigma chemicals or Research Biochemicals. One outer barrel of micropipette was filled with 0.15 M NaCl for current balance. Retaining currents (5~10 nA) were used between drug application to prevent drug leakage. In each figure, the number next to the name of each substance is the amount of ejection current. For example, Ca^{2+} 5 means that Ca^{2+} was ejected at the current of 5nA. EAAs were iontophoretically applied for 5 sec every 15 or 20 sec. We first checked whether LT and WDR cells responded to iontophoretically applied EAAs and then observed the effects of Ca^{2+}, EGTA, Mn^{2+}, verapamil and ω-conotoxin GVIA on the responses evoked by EAAs. Also studied were the effects of Ca^{2+}, EGTA, $Mn^{2+}$and verapamil on the SP-induced changes in spontaneous activity and the responses of WDR cell to NMDA and kainate. In a few experiments, changes in the responses of WDR cell to the iontophoretic application of glutamate were observed after spinal application of EGTA (20 mM).

All evoked activities were amplified (WPI, DAM-80, USA), displayed on oscilloscope and fed into the window discriminator whose output was used by a computer to compile the poststimulus time histogram. Because each unit had different threshold current and size of the EAA-induced responses varied from one unit to another, data were expressed as a percentage of discharges in the control state before single or combined iontophoretic application of SP, Ca^{2+} and Ca^{2+} channel blockers.

RESULTS

The activity of 254 dorsal horn neurons was recorded in 64 experiments. The dorsal horn neurons included 215 WDR cells and 29 LT cells. Recordings

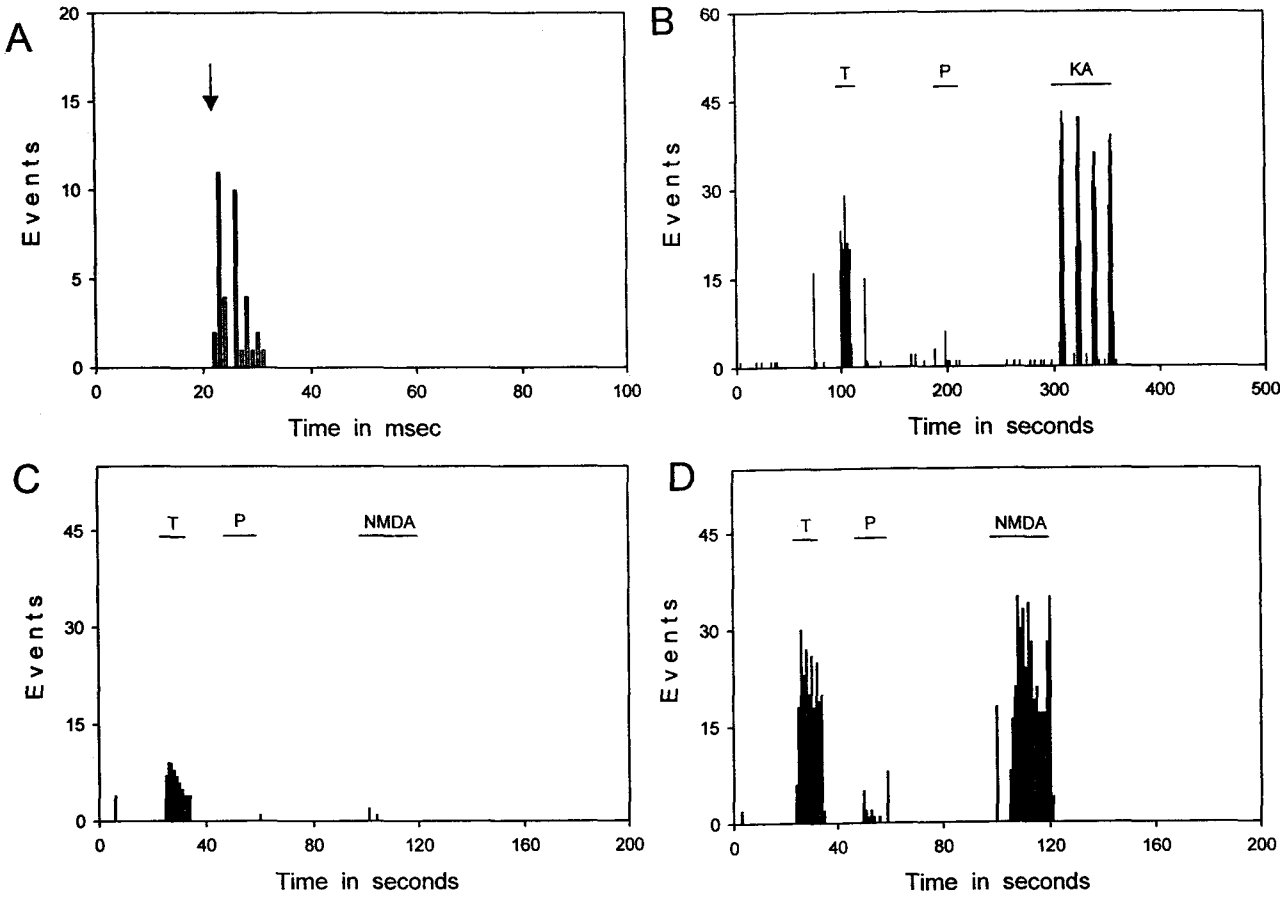

Fig. 1. Response characteristics of the low threshold (LT) cell to iontophoretical application of kainate and NMDA. T: tapping, P: pressure. Each mechanical stimulus was applied to the receptive field for 10 sec. The neurons tested responded only to tapping stimulus A: A-fiber response induced by electrical stimulation of afferent nerves. This neuron received only A-fiber inputs. Arrow in A indicate the time at which single stimulus was applied to afferent nerve. All LT cell (N=10) tested responded to kainate (B). 16/29 LT cells responded to iontophoretically applied NMDA or glutamate while 13/29 LT cells did not respond (C).

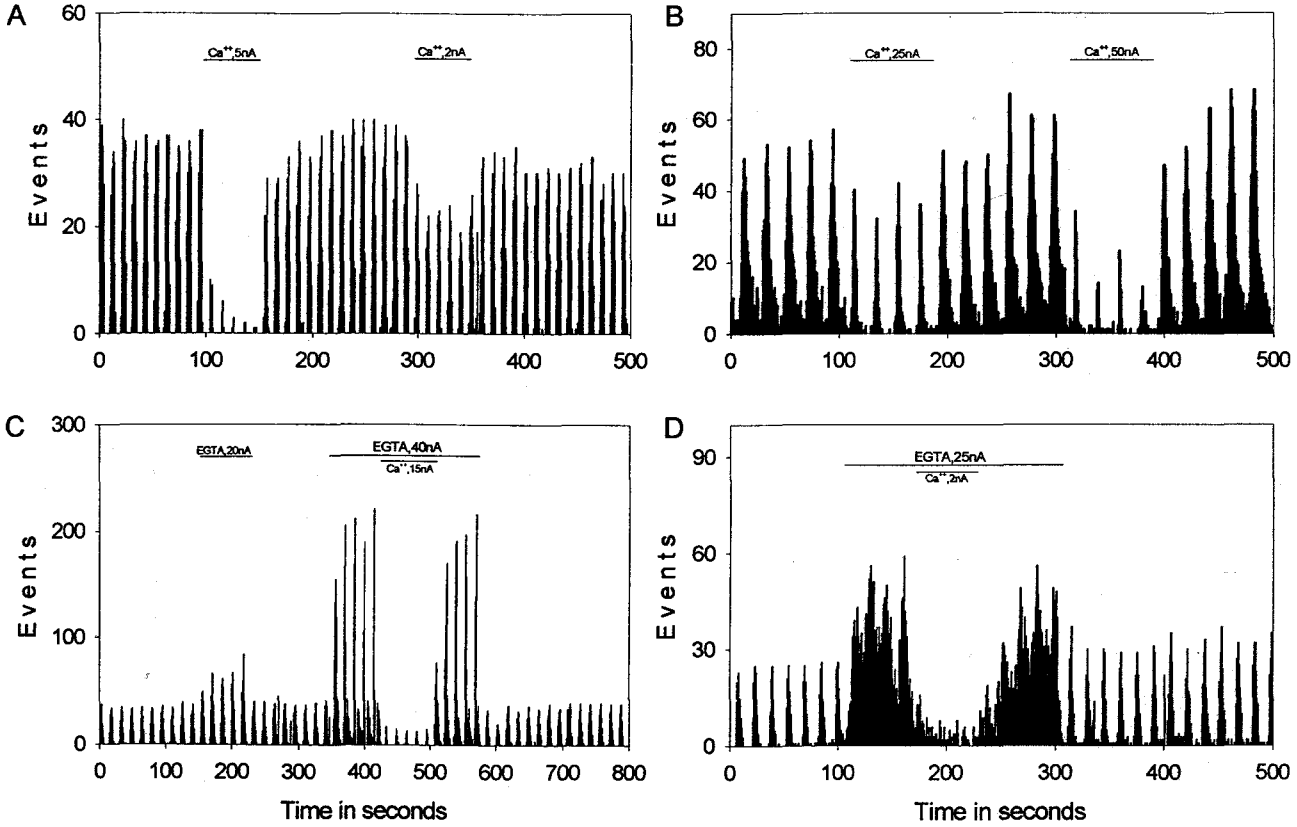

Fig. 2. Changes in the NMDA- and kainate- induced responses of wide dynamic range (WDR) cell after single or combined iontophoretical application of Ca^{2+} and EGTA. The NMDA- (A) and kainate- (B) induced responses of WDR cells were current-dependently inhibited by the iontophoretical application of Ca^{2+}. However, EGTA augmented the NMDA (C) and kainate- (D) induced responses and the augmenting action of EGTA was antagonized by Ca^{2+}.

were generally obtaind from 500 μm to 1000 μm below the surface of the spinal cord, suggesting that the cell bodies were located in laminae IV - VI. The LT cells tested responded only to tappings applied to the receptive field without any response to pressure. Of the 29 LT cells, 16 cells (55.1%) responded to iontophoretically applied glutamate or NMDA whereas 13 cells did not. However, all the LT cells tested (N=10) showed the response to kainate. The LT cells that responded to excitatory amino acids (EAA) had the same response characteristics to Ca^{2+} and Ca^{2+} channel blockers as those of WDR cells, and so figures were not given to avoid the repeated presentation of the figures with the same pattern. Of the 215 WDR cells recorded, the response characteristics of 114 WDR cells were more carefully investigated. 81 WDR cells received C-fiber inputs. Only 7/114 units did not respond to NMDA, glutamate or kainate. No differences in the EAAs-induced responses were observed between WDR cells with and without C-fiber inputs. Glutamate has been known to have a similar affinity both to NMDA and non-NMDA receptors but we could not find apparent differences in the responses to NMDA and glutamate in this study and both data were pooled.

Iontophoretically applied Ca^{2+} current-dependently suppressed both NMDA- and kainate-induced responses of WDR cells (Fig. 2A & B) while Ca^{2+} chelating agent, EGTA, strongly augmented the responses to NMDA and kainate (Fig. 2C & D). The augmenting effect of EGTA was greatly reduced by Ca^{2+}. A decisive data are not presented here because there was a great difference in threshold current for each WDR cell (this principle is applied to all other cases). Ca^{2+} ejected at the current of 5 nA inhibited NMDA-induced responses to about $49.8 \pm 7.5\%$ of the control and iontophoretic application of EGTA with the ejection current of 20 and 40nA augmented NMDA-induced responses to about $166.4 \pm 14.8\%$ and $239.8 \pm 22.5\%$ of the control, respectively.

Fig. 3. shows inhibitory effect of non-selective voltage-dependent Ca^{2+} channel blocker, Mn^{2+}, on the NMDA-induced responses. Inhibitory action of Mn^{2+} on the WDR cell responses to NMDA was current-dependent. When Mn^{2+} was iontophoretically applied at the ejection current of 10 nA, NMDA-

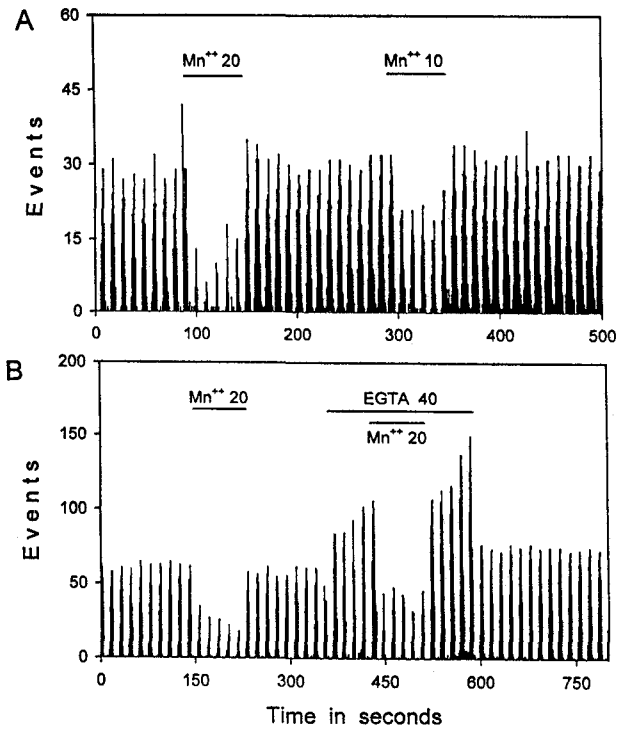

Fig. 3. Effects of iontophoretically applied manganese ions on the NMDA-induced responses of wide dynamic range (WDR) cells. Manganese ions current-dependently suppressed the NMDA-induced responses of WDR cells (A). EGTA-evoked augmentation of NMDA-induced responses was antagonized by manganese ions (B).

induced response was attenuated to 57.4±5.2% of the control. Mn^{2+} also suppressed the augmenting effect of EGTA on the NMDA-induced responses of WDR cell to 24.3±7.1% of the EGTA-augmented response.

In a few experiments (N=5), we observed the effect of EGTA applied onto the spinal cord for a longer duration (Fig. 4). For about 700~800 sec after spinal application of EGTA, the responses of WDR cell to iontophoretical application of NMDA were augmented and thereafter gradually inhibited. The NMDA-evoked responses were almost completely suppressed about 1200 sec after spinal application of EGTA.

In Fig. 5, inhibitory effects of N-type and L- type Ca^{2+} channel blockers on the glutamate- and kainate-induced responses of WDR cells are shown. In general, N-type Ca^{2+} channel blocker, ω-conotoxion GVIA, had weaker inhibitory action and needed higher ejection current for inhibition (200~300 nA) (Fig. 5A & C). Therefore, we observed mainly the effect of L-type Ca^{2+} channel blocker in the following experiments. ω-conotoxin inhibited NMDA-induced responses by about 60.4±5.4% when ejected at the current of 200 nA. However, L-type Ca^{2+} channel blocker, verapamil, had strong and long-lasting inhibitory action on the EAA-induced responses of WDR cell. The NMDA - induced response was suppressed to about 34.4±10.3% of the control by verapamil ejected at the current of 15~20 nA and also kainate-induced responses were inhibited to a similar degree.

Substance P (SP) had variable actions on the

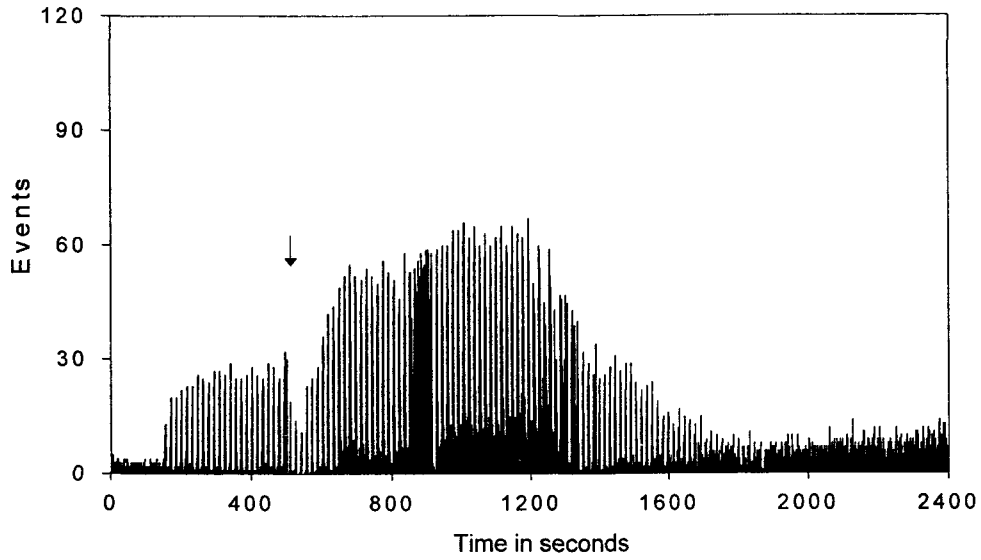

Fig. 4. Effects of spinal application of EGTA on the NMDA-induced responses of wide dynamic range (WDR) cells. WDR cells were excited by iontophoretical application of NMDA for 5 sec every 20 sec. EGTA (20 mM) was applied onto the spinal cord at arrow.

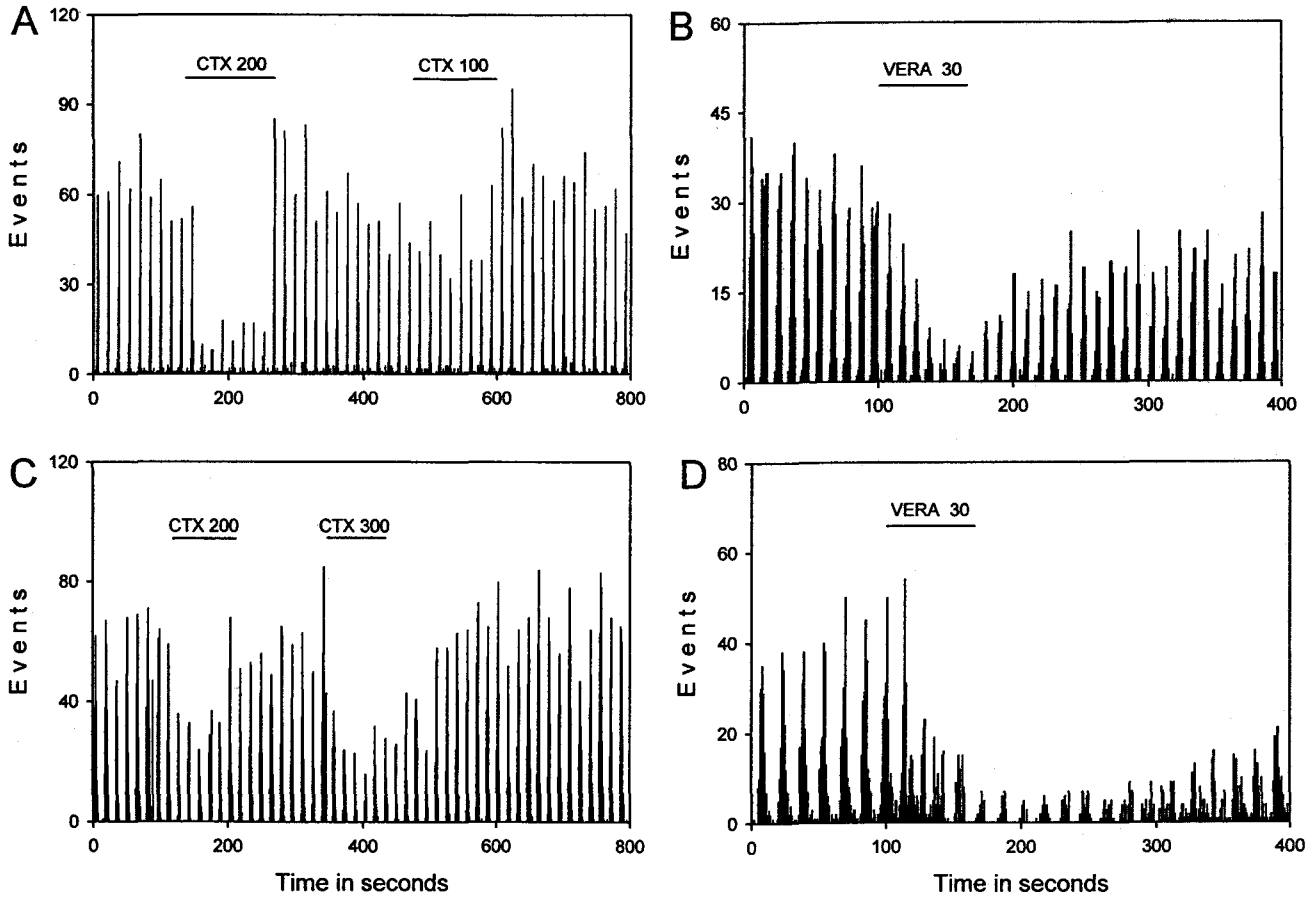

Fig. 5. Changes in the NMDA- and kainate-induced responses of wide dynamic range (WDR) cell after iontophoretical application of ω-conotoxin GVIA (CTX) and verapamil (VERA). Both responses induced by NMDA (A) and kainate (C) were inhibited by CTX but a little high current was required for the induction of inhibition. However, verapamil induced a long-lasting inhibition of the responses of WDR cells to NMDA (B) and kainate (D).

NMDA- and kainate-induced responses of WDR cells. Predominant action of SP was excitatory on the NMDA-induced response. Action of SP had very slow onset and offset. The excitatory effect of SP reached the peak level at least 60 sec after beginning iontophoretical application of SP. Of the 66 cells tested, following the iontophoretical application of SP at the ejection current of 50 nA, the NMDA-induced response of WDR cell was augmented to 185.9± 9.9% (115.5~274.9%) of the control in 35 cells and was inhibited to 54.9±4.8% of the control in 13 cells. In remaining 18 cells, the NMDA-induced responses were not changed by SP. SP had a similar effect on the kainate-induced responses. However, kainate-induced responses were inhibited in a higher proportion of WDR cells (13/30 cells) whereas the responses were potentiated in 8/30 cells by SP. Figure 6 shows typical examples of the NMDA-induced responses of WDR cell which were excited or inhibited by iontophoretical application of SP.

Because we could not find any difference in the effects of Ca^{2+} and Ca^{2+} channel blockers on the excitatory action of SP on NMDA- and kainate-induced responses, we describe only NMDA-induced response. As shown in Fig. 7, iontophoretically applied Ca^{2+}, Mn^{2+} and verapamil inhibited the SP-augmented responses of WDR cells by 70.4±5.2%, 74.3±6.5% and 73.2±4.6%, respectively. Inhibitory actions of Ca^{2+} and Mn^{2+} were short-lasting but that of verapamil was very long-lasting. The excitatory effect of SP was further increased after iontophoretical administration of EGTA (Fig. 7B).

We examined the effects of SP on the spontaneous activities in 25 WDR cells. SP augmented the spontaneous activities by 78.2±22.6% in 13 cells while inhibited by 69.2±6.2% in 6 cells (Fig. 8A & B). The spontaneous activities were not changed in 6 cells even after iontophoretical application of SP. Excitatory effect of SP on spontaneous activity was also suppressed by Ca^{2+}, Mn^{2+} and verapamil. The action of verapamil was very strong and long-lasting (Fig. 8C & D).

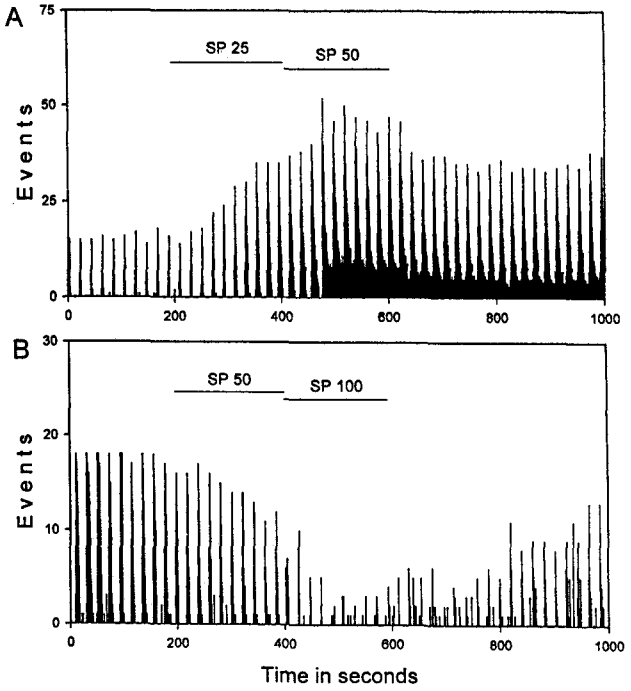

Fig. 6. Typical examples of the NMDA-induced responses of WDR cells which were excited or inhibited by iontophoretical application of substance P (SP). The NMDA-induced responses were more strongly excited (A) or inhibited (B) when ejection current increased. SP had very slow onset and offset.

DISCUSSION

Many experimental findings suggest that NMDA and SP are implicated in the processing of nociceptive informations in the spinal cord. The evidence which supports this contention includes 1) high density of their binding sites in superficial layers of dorsal horn where the nociceptive primary afferent fibers terminate (Henley et al, 1993; Marshall et al, 1996), 2) an increase in the release of SP and NMDA from capsaicin-sensitive fine afferent fibers by the noxious stimulation (Duggan et al, 1988; Kangrga et al, 1990; Kangrga & Randić, 1991), and 3) the excitation by NMDA and SP of dorsal horn neurons which receive noxious inputs from the peripheral receptors and are located mainly in laminae I and II (Randić & Miletic, 1977; Schneider & Perl, 1985). The evoked release of SP and EAA greatly increases in the spinal cord when cutaneous inflammation and arthritis are induced by formalin (Skilling et al, 1988), carrageenan (Schaible et al, 1990) and complete Freund's adjuvant (Southall et al, 1998). The SP and EAA receptor antagonists also suppress the inflammation-induced and neuropathic pains (Seltzer et al, 1991; Mao et al, 1992; Sluka & Westlund, 1993; Traub, 1996). All these results imply that SP and EAA may play a key role in the nociceptive sensory transmission in the spinal cord. In this experiment, we found that LT cells as well as WDR cells are excited by iontophoretically applied NMDA and kainate and that LT cells activated by EAAs had the same response characteristics to Ca^{2+} and Ca^{2+} channel blockers as those of WDR cells. Our finding that LT cells were excited by EAA does not agree with the view that EAA functions as a nociceptive neurotransmitter. Although we can not reach a definite conclusion from the result of this experiment, the activation of LT cells as well as WDR cells by EAA seems to be related with the localization of the site stimulated by noxious stimulations.

The EAA-induced responses of WDR cell were current-dependently inhibited by iontophoretically applied Ca^{2+} while they were augmented by EGTA. The augmented WDR cell responses to EGTA were suppressed by Ca^{2+} or Mn^{2+}. These results are in a sharp contrast with other worker's fingings that intracerebroventricular (i.c.v.) administration of Ca^{2+} produced hyperalgesia while i.c.v. or intrathecal EGTA induced dose-dependent analgesia (Schmidt & Way, 1980; Kim et al, 1991). On the other hand, we observed biphasic effects of EGTA on NMDA responses after spinal application: initial augmentation and later suppression. For about 700~800 sec after spinal application of EGTA the NMDA responses of WDR cells were potentiated and thereafter the augmented responses were attenuated. These differences in the effects of Ca^{2+} and EGTA can be attributed to how much changes in Ca^{2+} concentration in ECF were induced by iontophoretical or spinal application of Ca^{2+} or EGTA. Local and small increase in Ca^{2+} concentration by iontophoretical application of Ca^{2+} can reduce the membrane excitability whereas low Ca^{2+} concentration can decrease the threshold for excitation and then increase the spontaneous discharges (Frankenhaeuser, 1957; Frankenhaeuser & Meves, 1958). Curtis et al (1960) also reported that iontophoretical application of calcium chelators excited spinal neurons by reducing Ca^{2+} concentration in ECF and by removing Ca^{2+} combined with membrane protein. However, the NMDA responses of WDR cells in this study appears to be strongly suppressed because Ca^{2+} concentration in both ECF and ICF was greatly reduced after longer spinal application of EGTA. Iontophoretical or spinal application of calcium ions and EGTA seem to induce nonspecific action on the spinal neurons which vary

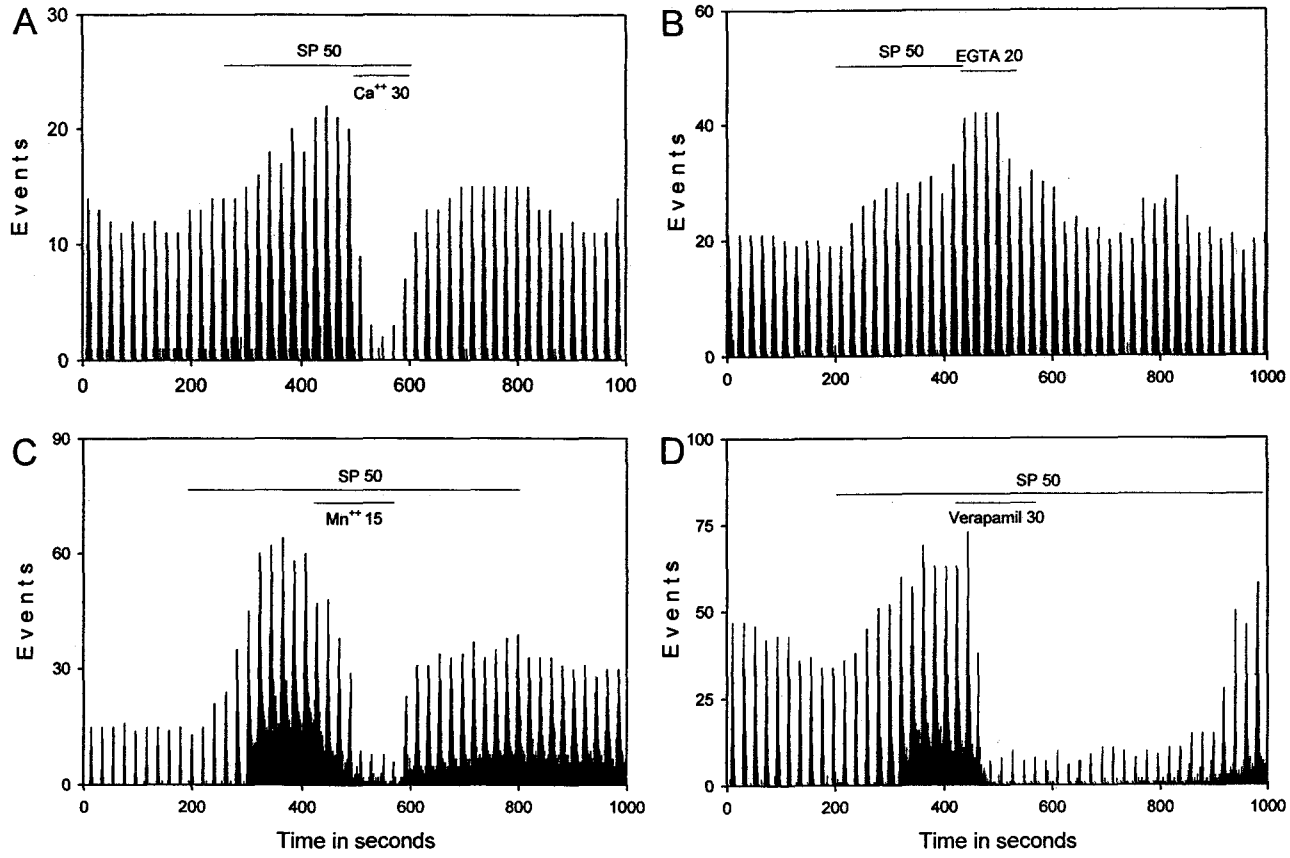

Fig. 7. Ca^{2+}, EGTA, Mn^{2+} and verapamil modified the NMDA-induced responses of WDR cell which were augmented by iontophoretical application of substance P (SP). Ca^{2+} (A), Mn^{2+} (C) and verapamil (D) strongly suppressed SP-evoked augmentation of the responses of WDR cell to NMDA whereas EGTA further increased augmenting effect of SP (B).

with the induced changes of calcium concentration in ECF and neuronal membrane.

Ca^{2+} influx through voltage sensitive Ca^{2+} channels as well as EAA receptor is assumed to play an important role in the EAA-induced responses of WDR cells. The responses of WDR cells to NMDA and kainate were suppressed by iontophoretical application of Mn^{2+}, verapamil and ω-conotoxin GVIA. Of these three Ca^{2+} channel blockers, verapamil produced most strong and sustained inhibition of the EAA-induced responses of WDR cells. EAA has been known to induce an increase in $[Ca^{2+}]_i$ in hippocampal, striatal and spinal neurons, which was antagonized by AP5 (2-amino-5-phosphonovalerate) and removal of external Ca^{2+} (Murphy et al, 1987; Mayer et al, 1987). Murphy et al (1987) reported that the NMDA- and kainate-induced increase in $[Ca^{2+}]_i$ was not blocked by lanthanum, a non-selective voltage-dependent Ca^{2+} channel blocker. These experimental findings suggest that Ca^{2+} enters the neuronal cell only through NMDA receptors and in this respect do not agree with our results, that calcium entry through voltage sensitive calcium channels, especially L-type, played an important role in the NMDA- and kainate-induced responses of WDR cells.

As reported by others, SP had triple effects on spontaneous activity and EAA-evoked responses of WDR cells in the present study. The mechanism by which SP induces an inhibitory action remains to be clarified. Excitatory action of SP on the EAA responses has been studied in behavioral and electrophysiological experiments (Smullin et al, 1990; Dougherty et al, 1993). In the present study, excitatory action of SP was observed in more than 50% of WDR cells activated by NMDA or those cells with spontaneous activities. This excitatory action was strongly suppressed by iontophoretic application of Ca^{2+}, Mn^{2+} and verapamil, suggesting that Ca^{2+} entry through voltage sensitive Ca^{2+} channels plays an important role in the excitatory effect of SP on the NMDA responses. In a single isolated rat dorsal horn neuron, $[Ca^{2+}]_i$ of dorsal horn neuron activated by SP was increased and the increase in $[Ca^{2+}]_i$ was

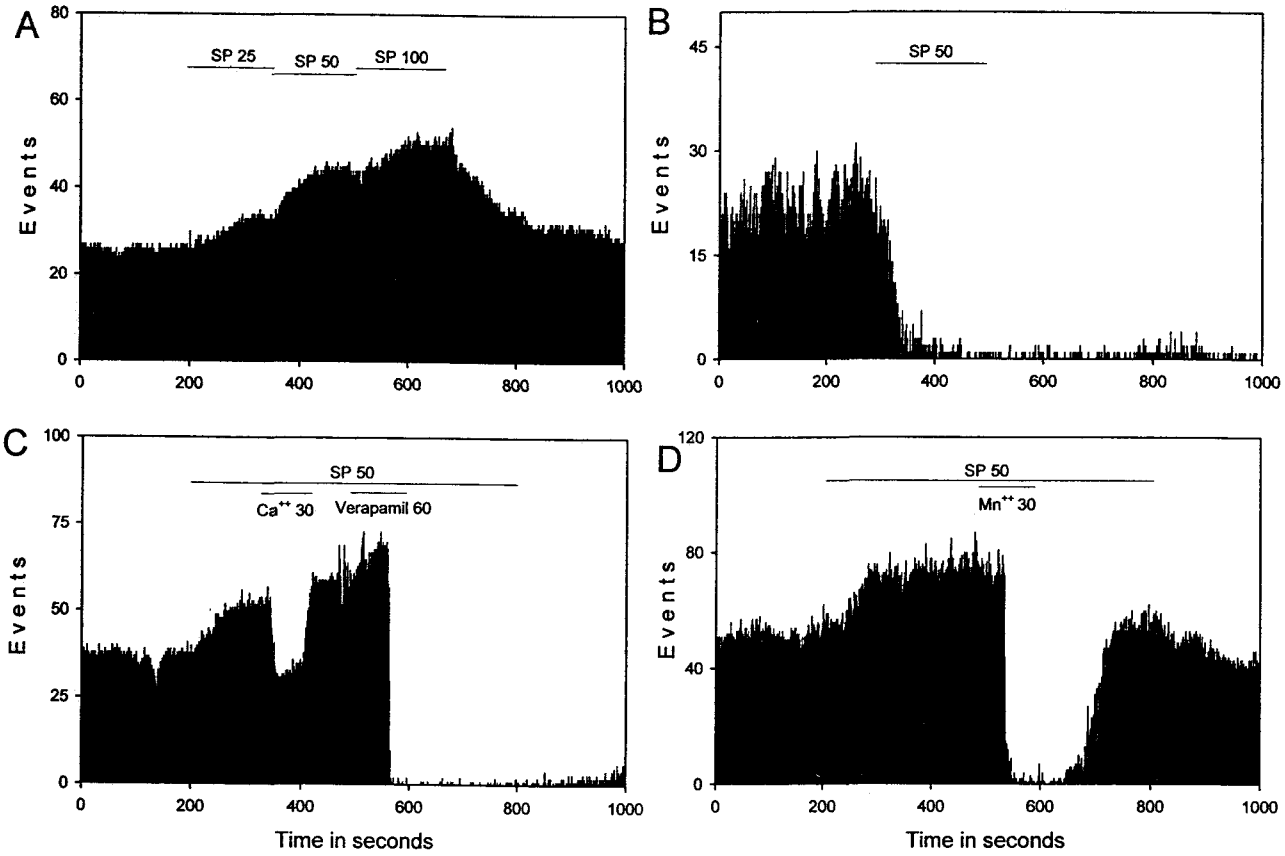

Fig. 8. Changes in the spontaneous activities after single or combined administration of substance P (SP), Ca^{2+}, verapamil and Mn^{2+}. Iontophoretical application of SP increased (A) or decreased (B) the spontaneous activities of WDR cells. The increased spontaneous activities by SP were attenuated after iontophoretical application of Ca^{2+} (C), verapamil (C) and Mn^{2+}(D).

antagonized by $LaCl_3$, verapamil and Ca^{2+} free medium (Heath et al, 1994; Murase et al, 1986). These results well agree with our finding that the SP-induced excitatory effects were strongly antagonized by Ca^{2+} channel blockers, especially L-type blocker.

The EAA- and SP-induced increase in $[Ca^{2+}]_i$ may cause 1) an activation of protein kinase C which induces sustained alternations in the cellular membrane, 2) the production of inositol 1,4,5-trisphosphate (IP_3) which causes further release of calcium from intracellular stores within the endoplasmic reticulum, 3) the formation of arachidonic acid products by the activation of phospholipase A_2, and 4) the activation of nitric oxide (NO) synthase and liberation of NO (Coderre, 1992; Rodriguez-Alvarez et al, 1996). These factors act together and then may lead to the central sensitization and hyperalgesia. For the following reasons, authors believe that SP may function as a neuromodulator rather than neurotransmitter in the processing of nociceptive informations in the spinal cord. SP and EAA are colocalized in the same terminals of primary afferent fibers (Battaglia & Rustioni, 1988; De Biasi & Rustioni, 1988) and they are released simultaneously by the same noxious stimuli (Duggan et al, 1988; Kangrga & Randić, 1991; Paleckova et al, 1992). EAA has rapid onset and offset while SP induced slow and prolonged depolarization. In the present study, more than 60 sec was needed for the effect of SP to reach the peak after iontophoretic application and the SP-evoked effect was very long-lasting. It was well documented that SP increases the release of EAA from primary afferent fiber (Smullin et al, 1990), and also augments behavioral and electrophysiological responses induced by EAA (Smullin et al, 1990; Dougherty et al, 1993). Putting all these reports together, we can conclude that EAA with rapid onset first excites dorsal horn neurons and then the evoked responses of dorsal horn neuron and the release of EAA are continuously potentiated by SP with slow onset. These mutual action of SP and EAA can lead to the sustained activation of dorsal horn neurons.

ACKNOWLEDGEMENT

The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1997.

REFERENCES

Battaglia G, Rustioni A. Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. *J Comp Neurol* 277: 302−312, 1988

Chaplan SR, Pogrel JW, Yaksh TL. Role of voltage-dependent calcium channel subtypes in experimental tactile allodynia. *J Pharmacol Exp Ther* 269: 1117−1123, 1994

Coderre TJ. Contribution of protein kinase C to central sensitization and persistent pain following tissue injury. *Neurosci Lett* 140: 181−184, 1992

Curtis DR, Phillips TW, Watkins JC. Chemical excitation of spinal neurones. *Nature* 183: 611−612, 1959

Curtis DR, Phillips TW, Watkins JC. The excitation of spinal neurones by the ionophoretic application of agents which chelate calcium. *J Neurochem* 6: 1−20, 1960

De Biasi S, Rustioni A. Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc Natl Acad Sci USA* 85: 7820−7824, 1988

De Koninck Y, Ribeiro-Da-Silva A, Henry JL, Cuello AC. Spinal neurons exhibiting a specific nociceptive response receive abundant substance P-containing synaptic contacts. *Proc Nate Acad Sci USA* 89: 5073−5077, 1992

Dougherty PM, Palecek J, Zorn S, Willis WD. Combined application of excitatory amino acids and substance P produces long-lasting changes in responses of primate spinothalamic tract neurons. *Brain Res Rev* 18: 227−246, 1993

Duggan AW, Hendry IA, Morton CR, Hutchison WD, Zhao ZQ. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. *Brain Res* 451: 261−273, 1988

Fleckenstein A, Frey M, Zorn J, Fleckenstein-Grün G. Experimental basis of the long-term therapy of arterial hypertension with calcium antagonists. *Am J Cardiol* 56: 3H−14H, 1985

Frankenhaeuser B. The effect of calcium on the myelinated nerve fibre. *J Physiol* 137: 245−260, 1957

Frankenhaeuser B, Meves H. The effect of magnesium and calcium on the frog myelinated nerve fibre. *J Physiol* 142: 360−365, 1958

Gürdal H, Sara Y, Tulunay FC. Effects of calcium channel blockers on formalin-induced nociception and inflammation in rats. *Pharmacology* 44: 290−296, 1992

Heath MJS, Womack MD, MacDermott AB. Substance P elevates intracellular calcium in both neurons and glial cells from the dorsal horn of the spinal cord. *J Neurophysiol* 72: 1192−1198, 1994

Henry JL, Yashpel K, Reid J, Samulack DD. Calcium mediation of hyperalgesic effects of substance P in rat spinal cord. *Soc Neurosci Abst* 22: 875, #345.9, 1996

Jessell T, Tsunoo A, Kanazawa I, Otsuka M. Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. *Brain Res* 168: 247−259, 1979

Kangrga I, Larew JSA, Randić M. The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. *Neurosci Lett* 108: 155−160, 1990

Kangrga I, Randić M. Outflow of endogenous aspartate and glutamate from the rat spinal dorsal horn in vitro by activation of low- and high- threshold primary afferent fibers: Modulation by μ-opioids. *Brain Res* 553: 347−352, 1991

Kim JH, Shin HK, Kim KS, Huh C. Analgesic action of calcium modulators in the cat. *J Hanyang Med Coll* 11: 464−473, 1991

Konishi S, Otsuka M. The effects of substance P and other peptides on spinal neurons of the frog. *Brain Res* 65: 397−410, 1974

Malmberg AB, Yaksh TL. Effect of continuous intrathecal infusion of ω-conopeptides, N-type calcium channel blockers, on behavior and antinociception in the formalin and hot-plate tests in rats. *Pain* 60: 83−90, 1995

Mao J, Price DD, Hayes RL, Lu J, Mayer DJ. Differential roles of NMDA and non-NMDA receptor activation in induction and maintenance of thermal hyperalgesia in rats with painful peripheral mononeuropathy. *Brain Res* 598: 217−278, 1992

Marshall GE, Shehab SAS, Spike RC, Todd AJ. Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. *Neuroscience* 72: 255−263, 1996

Mayer ML, MacDermott AB, Westbrook GL, Smith SJ, Barker JL. Agonist- and voltage-gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured using arsenazo III. *J Neurosci* 7: 3230−3244, 1987

Murase K, Ryu PD, Randić M. Substance P augments a persistent slow inward calcium-sensitive current in voltage-clamped spinal dorsal horn neurons of the rat. *Brain Res* 365: 369−376, 1986

Murphy SN, Thayer SA, Miller RJ. The effects of excitatory amino acids on intracellular calcium in a single mouse striatal neurons in vitro. *J Neurosci* 7: 4145−4158, 1987

Neugebauer V, Vanegas H, Nebe J, Rümenapp P, Schaible HG. Effects of N- and L- type calcium channel antagonists on the responses of nociceptive spinal cord neurons to mechanical stimulation of the

normal and the inflamed knee joint. *J Neurophysiol* 76(6): 3740−3749, 1996

Olivera BM. Calcium channel diversity and neurotransmitter release: The ω-contoxines and ω-agatoxins. *Ann Rev Biochem* 63: 823−867, 1994

Paleckova V, Palecek J, McAdoo DJ, Willis WD. The non-NMDA antagonist CNQX prevents release of amino acids into the rat spinal cord dorsal horn evoked by sciatic nerve stimulation. *Neurosci Lett* 148: 19−22, 1992

Raigorodsky G, Urca G. Involvement of N-methyl-D-aspartate receptors in nociception and motor control in the spinal cord of the mouse: behavioral, pharmacological and electrophysiological evidence. *Neuroscience* 36: 601−610, 1990

Randić M, Miletic V. Effects of substance P in cat dorsal horn neurones activated by noxious stimuli. *Brain Res* 128: 164−169, 1977

Rodriguez-Alvarez J, Blanco I, Patel AJ. Modulation of N-methyl-D-aspartate receptor mediated arachidonic acid release by endogenous nitric oxide in cultured neurons. *Neurosci Lett* 218: 95−98, 1996

Schaible HG, Jarrott B, Hope PJ, Duggan AW. Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microphrobes. *Brain Res* 529: 214−223, 1990

Schmidt WK, Way EL. Hyperalgesic effects of divalent cations and antinociceptive effects of a calcium chelator in naive and morphine-dependent mice. *J Pharmacol Exp Ther* 212: 22−27, 1980

Schneider SP, Perl ER. Selective excitation of neurons in the mammalian spinal dorsal horn by aspartate and glutamate in vitro: correlation with location and excitatory input. *Brain Res* 360: 339−343, 1985

Seltzer Z, Cohn S, Ginzburg R, Beilin B. Modulation of neuropathic pain behavior in rat by spinal disinhibition and NMDA receptor blockade of injury discharge. *Pain* 45: 69−75, 1991

Shin HK, Kang SH, Kim KS. Calcium channel blockers suppress the responses of rat dorsal horn cell to nociceptive input. *Kor J Physiol Pharmacol* 1: 625−637, 1997

Skilling SR, Smullin DH, Beitz AJ, Larson AA. Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following veratridine and nociceptive stimulation. *J Neurochem* 51: 127−132, 1988

Sluka KA, Westlund KN. Centrally administered non-NMDA but not NMDA receptor antagonists block peripheral knee joint inflammation. *Pain* 55: 217−225, 1993

Smullin DH, Skillin SR, Larson AA. Interactions between substance P, calcitonin gene-related peptide, taurine and excitatory amino acids in the spinal cord. *Pain* 42: 93−101, 1990

Southall MD, Michael RL, Vasko MR. Intrathecal NSAIDS attenuate inflammation-induced neuropeptide release from rat spinal cord slices. *Pain* 78: 39−48, 1998

Traub RJ. The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. *Pain* 67: 151−161, 1996

Zapater P, Javaloy J, Román JF, Vidal MT, Horge JF. Anticonvulsant effects of nimodipine and two novel dihydropyridines (PCA 50922 and PCA 50941) against seizures elicited by pentylenetetrazole and electroconvulsive shock in mice. *Brian Res* 796: 311−314, 1998

Zapater P, Moreno J, Horga JF. Neuroprotection by the novel calcium antagonist PCA 50938, nimodipine and flunarizine, in gerbil global brain ischemia. *Brain Res* 772: 57−62, 1997