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Responses of Dorsal Horn Neurons to Peripheral Chemical Stimulation in the Spinal Cord of Anesthetized Cats

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Although nociceptive informations are thought to be processed via different neural mechanisms depending on the types of stimuli, sufficient data have not been accumulated yet. We performed a series of experiments to elucidate the possible neural mechanisms as to chemical stimuli such as formalin, capsaicin and ATP. Single unit activity of wide dynamic range (WDR) neurons and high threshold cells were recorded extracellularly from the lumbosacral enlargement of cat spinal cord before and after chemical stimulation to its receptive field (RF). Each chemical substance - formalin (20 μl, 4%), capsaicin (33 mM) or Mg-ATP (5 mM)- was injected intradermally into the RFs and then the changes in the spontaneous activity, mechanical threshold and responses to the peripheral mechanical stimuli were observed. In many cases, intradermal injection of formalin (5/11) and capsaicin (8/11) resulted in increase of the spontaneous activity with a biphasic pattern, whereas ATP (8/8) only showed initial responses. Time courses of the biphasic pattern, especially the late response, differed between formalin and capsaicin experiments. One hour after injection of each chemical (formalin, capsaicin, or ATP), the responses of the dorsal horn neurons to mechanical stimuli increased at large and the RFs were expended, suggesting development of hypersensitization (formalin 6/10, capsaicin 8/11, and ATP 15/19, respectively). These results are suggested that formalin stimulates peripheral nociceptor, local inflammation and involvement of central sensitization, capsaicin induces central sensitization as well as affects the peripheral C-polymodal nociceptors and neurogenic inflammation, and ATP directly stimulates peripheral nociceptors.

Key Words: Formalin, Capsaicin, ATP, Wide dynamic, Range neuron, Dorsal horn neuron, Hypersensitization

INTRODUCTION

The noxious peripheral stimuli usually result in tissue damage. Once tissue is injured, a number of endogenous chemicals released from the damaged tissue can excite peripheral nociceptors and induce inflammation with pain sensation. The representative candidates for endogenous chemicals involved in this process are K^+ and ATP. K^+ released from damaged tissue plays an important role in determining the membrane potential and then subsequently can excite the nociceptors. In some studies, it was reported that intra-arterial injection of isotonic K^+ solution induced the pain sensation (Kanaka et al, 1985; Schaible & Schmidt, 1988). However, these actions of K^+ are not considered as the specific responses of nociceptive processing because the increment of extracellular K^+ concentration could generally depolarize membrane potential.

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Recently, ATP may also be known as endogenous chemical substance to be able to activate the nociceptor. $P2X_3$ is ATP-binding receptor known as a subtype of P2X purinergic receptors. This receptor was found in C-cell associated with nociception in dorsal root ganglion and it was also reported that ATP might excite nociceptor through cation channel, combined in a heteropolymer of $P2X_2$ and $P2X_3$ (Chen et al, 1995; Lewis et al, 1995; Burnstock & Wood, 1996).

In addition, H^+, bradykinin (BK), histamine, eicosanoids, noradrenalin, and serotonin (5-HT) released from injured tissues can also excite peripheral nociceptors (Reeh & Kress, 1995; Kress & Reeh, 1996). In the case of inflammation, for example, pH of exudates in the vicinity of injured site decreased from 7.4 to 5 so that this acidic condition could evoke pain sensation (Jacobus et al, 1977). It was also reported that BK, produced by enzyme activated by tissue damage, depolarized the membrane of the nociceptor via B_2 receptor and sensitized the nociceptor via PKC (Cesare & McNaughton, 1996). As indicated previously, these chemical substances released from the terminal of nociceptor, immune cells, and/or the damaged cells excited nociceptors and then induced hyperalgesia.

The understanding of the neural mechanisms for pain has advanced considerably but has not investigated whether the pain responses to various chemical stimuli are mediated by the same mechanisms of processing of nociceptive information.

A lot of behavioral methods have been developed in order to study nociception in animals. The formalin test is increasingly used as a model of injury-induced pain. In behavioral responses (flinching, licking, biting or scratching the injected paw, Almeida et al, 1999) observed in rodents, pain evoked by formalin appeared as biphasic states (Dubuisson & Denis, 1977). The previous study indicated that the early response might be induced by direct stimulation of formalin to nociceptor and the late response might be induced by both central sensitization and acute inflammation of tissue after formalin injection (Dickenson & Sullivan, 1987).

Nociceptors are characterized, at least partly, by their sensitivity to capsaicin (8-methyl-N-vanillyl-6-nonenamide)- the main pungent ingredient in hot chilly peppers- which elicits a sensation of burning pain by selectively activating neurons that transfer nociceptive information from the peripheral to the central nervous system (Caterina et al, 1997).

Capsaicin excited selectively C-polymodal nociceptors. Capsaicin, within the range of several mM, injected intradermally into human might evoke the responses similar to burning sensation or flare response (Simone et al, 1989). The previous data also reported that the intradermal application of capsaicin into neonate rat resulted in the selective destruction of C-fiber (Scadding, 1980) and mechanical and/or thermal hyperalgesia were followed by injection of capsaicin within several seconds. These responses evoked by capsaicin may act through vanilloid receptors, temperature-sensitive cation channels (Kirschstein et al, 1999). It has also been reported that capsaicin receptor, ligand-gated non-selective cation channel (Acs et al, 1994; Wood & Docherty, 1997), was associated with proton channel (Bevan & Geppetti, 1994). All these suggest that various chemicals may have different nociceptive mechanisms. In present study, we investigated the responses of dorsal horn cells to various noxious chemical stimuli formalin, capsaicin, or ATP- and the processing mechanisms of chemical stimuli.

METHODS

Preparation of animal

A total of thirty cats irrespective of sex (body weight, 1.8~3.0 kg) were used. After pretreatment with atropine sulfate (0.2 mg/kg, s.c.) and ketamine hydrochloride (Ketalar, Yu-Han, Korea, 30 mg/kg, i.m.), the animal was anesthetized with a-chloralose (60 mg/kg, i.v.). Pancuronium bromide (Mioblock, Organon, Netherland; initial dose 0.4 mg, maintenance dose 0.4 mg/hr) was administered to relax systemic musculature and the animal was artificially ventilated with end-expiratory carbon dioxide concentration maintained in the range of 3.5~4.5% (Normocap CO_2 & O_2 monitor, Datex, Finland). Rectal temperature was monitored and maintained within 37.5°C by an electrical blanket (Hoemothermic Blanket Control Unit, Harvard Apparatus, U.S.A.). Arterial blood pressure was monitored and Hartmann solution was infused continuously.

Lumbosacral enlargement was exposed by a laminectomy done on L_2-S_2 vertebrae. After L_7-S_1 dorsal root was identified, several small pia holes were made for insertion of recording electrode. Then animal was

transferred to a stereotaxic apparatus. A warm mineral oil pool was made using skin flaps and the pool was maintained warm by a heating coil through which warm water was circulated. Animal was recovered for an hour or longer before experiment.

Stimulation and recording

The activities of dorsal horn cell to the noxious chemical stimuli applied to peripheral receptive field were recorded by using carbon-filament microelectrode. Microelectrodes of low resistance (1~3 MΩ), pulled by electrode puller (PE-2, Narishige), were used.

Single unit activities picked up by recording electrode were amplified through an AC amplifier (DAM 80, WPI, U.S.A.). The signals were displayed on an oscilloscope and fed into a window discriminator connected to a laboratory interface (CED 1401, Cambridge Electronic Design, U.K.). Through a laboratory interface the signals were stored and analyzed by personal computer to allow sampling and analysis of the spontaneous and evoked neuronal activity.

Experimental procedure

When a single neuronal activity of sufficient amplitude was identified, the cell was characterized by responses to graded mechanical stimuli applied to the receptive field. Usually the depth of recording site was in 500~1,200 μm and 1,500~3,000 μm. Threshold for mechanical stimuli was determined by von Frey filament. Here, the threshold means the intensity of von Frey filament, which induces the minimal response of dorsal horn cell. After identifying dorsal horn neuronal activity, natural noxious chemical stimuli (brush, pressure, pinch, and squeeze) were applied to hind paw of cat. The results were analyzed by compiling single pass time histograms.

Statistical analysis of data

Animal was euthanized with excessive dose of anesthetics at the end of experiment. All the data were expressed as percents of the control.

RESULTS

The spikes of each WDR cell (n=34) that showed gradual responses to both innocuous and noxious stimuli were recorded extracellularly from the lumbosacral enlargements of spinal cord in each adult cat (n=34). All recorded cells had the receptive fields (RF) responsive to mechanical stimuli on the ipsilateral hind paw or footpad. Most single unit activities were recorded at depths between 1000~3000 μm (laminae IV-VI of Rexed).

The responses of dorsal horn cell after formalin injection

Injection of diluted formalin (20 μl, 4%) into peripheral RF increased the spontaneous activity of cells. In vehicle (20 μl, 0.9% saline) injected to RF, there were no significant changes of single unit electrical activity. Some cells (6/11) showed only the increased spontaneous neuronal activity for initial hundreds of seconds after injection of formalin (Fig. 1Aa, b). The others (5/11), however, showed distinctive biphasic patterns, which had both initial response to be evoked immediately after formalin injection and delayed response revealed about 30 min after injection of formalin (Fig. 1Aa). These time courses of biphasic pattern were similar to the results of other behavioral studies previously reported (Dubuisson & Dennis, 1977; Detweiler, 1995).

Fig. 1B shows mechanical hyperalgesia induced by formalin injection. As strength of von Frey filaments gradually increased (29, 76, 126, 447 g), each response before formalin injection showed 12, 30, 28, 138 impulses/10 sec, respectively. And the single unit activity increased at the strength of each von Frey filament (95, 185, 234, 418 impulses/10 sec) one hour after formalin injection. The threshold for von Frey filaments also lowered from 126 g to 29 g in addition to the increase of the firing rates for 20 sec in resting state, which means the inflammation-induced sensitization of RF. These results suggest that the injection of formalin into RF induces hyperalgesia, allodynia. The responses to natural mechanical stimuli (brush, pressure, pinch, and squeeze) also largely enhanced from 43, 106, 196, 413 to 100, 191, 639, 1246 (impulses/10 sec), respectively. Six of ten cells, as a whole, showed mechanical hyperalgesia following intradermal formalin injection, whereas four cells revealed the decrease of spontaneous activity and responses to noxious stimuli after formalin injection (data not shown).

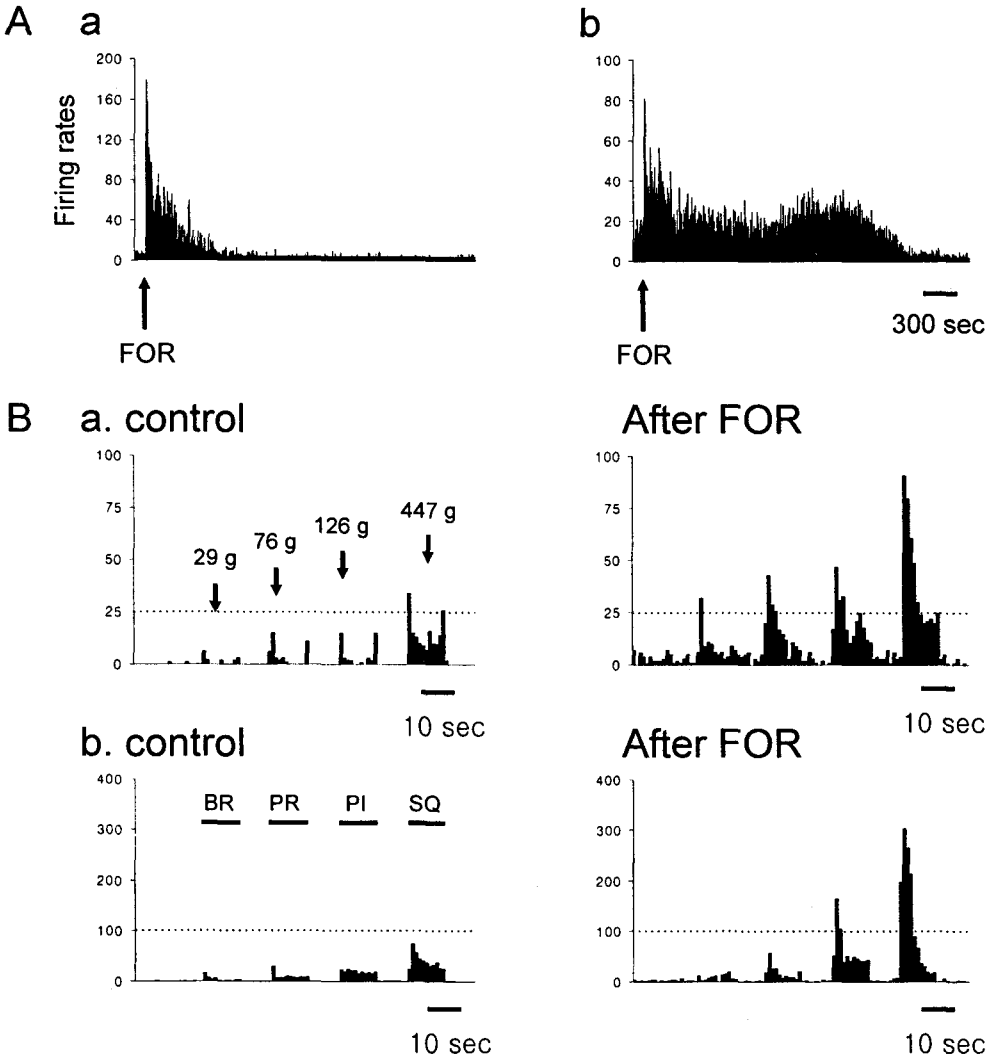

Fig. 1. Examples of responses of the dorsal horn neurons to formalin injection and peripheral noxious mechanical stimuli. Single cell activities were recorded from the lumbosacral area using an extracellular carbon-filament electrode. (A) a, b: Intradermal application of formalin (20 μl, 4%) resulted in increase of the spontaneous activity (a: monophasic pattern, b: biphasic pattern). (B) Responses of the dorsal horn neuron to mechanical stimuli increased after formalin injection. Ba: von Frey filament, Bb: natural mechanical stimuli. FOR; formalin, BR; brush, PR; pressure, PI; pinch, SQ; squeeze.

The responses of dorsal horn cell after capsaicin injection

The spontaneous activity increased immediately after intradermal injection of 20 μl capsaicin into peripheral RF, that is initial response, and then decreased to basal level within 100 sec (Fig. 2Aa, b). After vehicle injection into RF, single unit spontaneous activity did not change. In some cases (8 of 11 cells), we observed biphasic patterns as like formalin-evoked initial and delayed phase (Fig. 2Ab). The time courses of delayed-response, however, seemed to differ from the results of formalin-evoked responses. The cells responsive to capsaicin had two patterns of the delayed response; rapid-delayed response observed between 150 and 350 sec and slow-delayed response in 700 sec, respectively. Other phase seemed to appear between initial phase and delayed phase, which means triphasic pattern

Injection of capsaicin into RF also induced me-

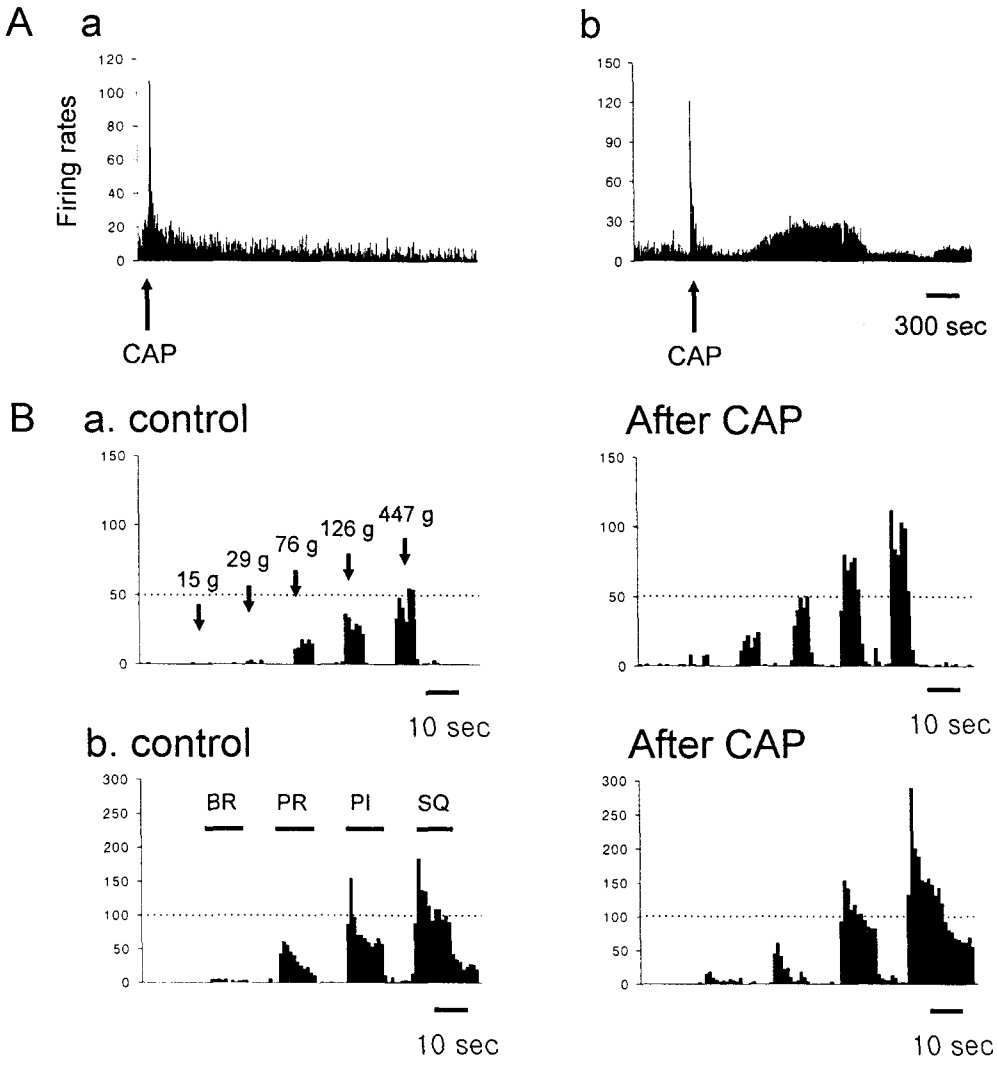

Fig. 2. Examples of responses of the dorsal horn neurons to capsaicin injection and peripheral noxious mechanical stimuli. (A) a, b: Intradermal application of capsaicin (20 μl, 33 mM) resulted in increase of the spontaneous activity (a: monophasic pattern, b: biphasic pattern). (B) Responses of a dorsal horn neuron to mechanical stimuli increased after capsaicin injection. Ba: von Frey filament, Bb: natural mechanical stimuli.

chanical hyperalgesia and reduced the threshold for mechanical stimuli. In Fig. 2Ba, the threshold of this single unit activity was 76 g intensity of von Frey filament. After capsaicin injection this cell also showed evoked-spontaneous activities in addition to the lower threshold (29 g) than that of control one hour later. Fig. 2Bb shows the response to natural mechanical stimuli before and after capsaicin injection. In this cell, the response to pressure decreased after capsaicin injection. In most cells, however, the response to pressure was enhanced by capsaicin injection. These increments in responses to innocuous and noxious stimuli were observed in 8 of 11 cells. In three cells, although their responses to noxious stimuli were decreased, the responses to innocuous stimuli also increased after capsaicin injection.

The responses of dorsal horn cell after ATP injection

Intradermal injection of ATP (20 μl, 5 mM) only induced the initial responses of dorsal horn neurons (n=8) unlike biphasic patterns shown by formalin or capsaicin. Injection of vehicle has no significant changes of single unit electrical activity. The delayed

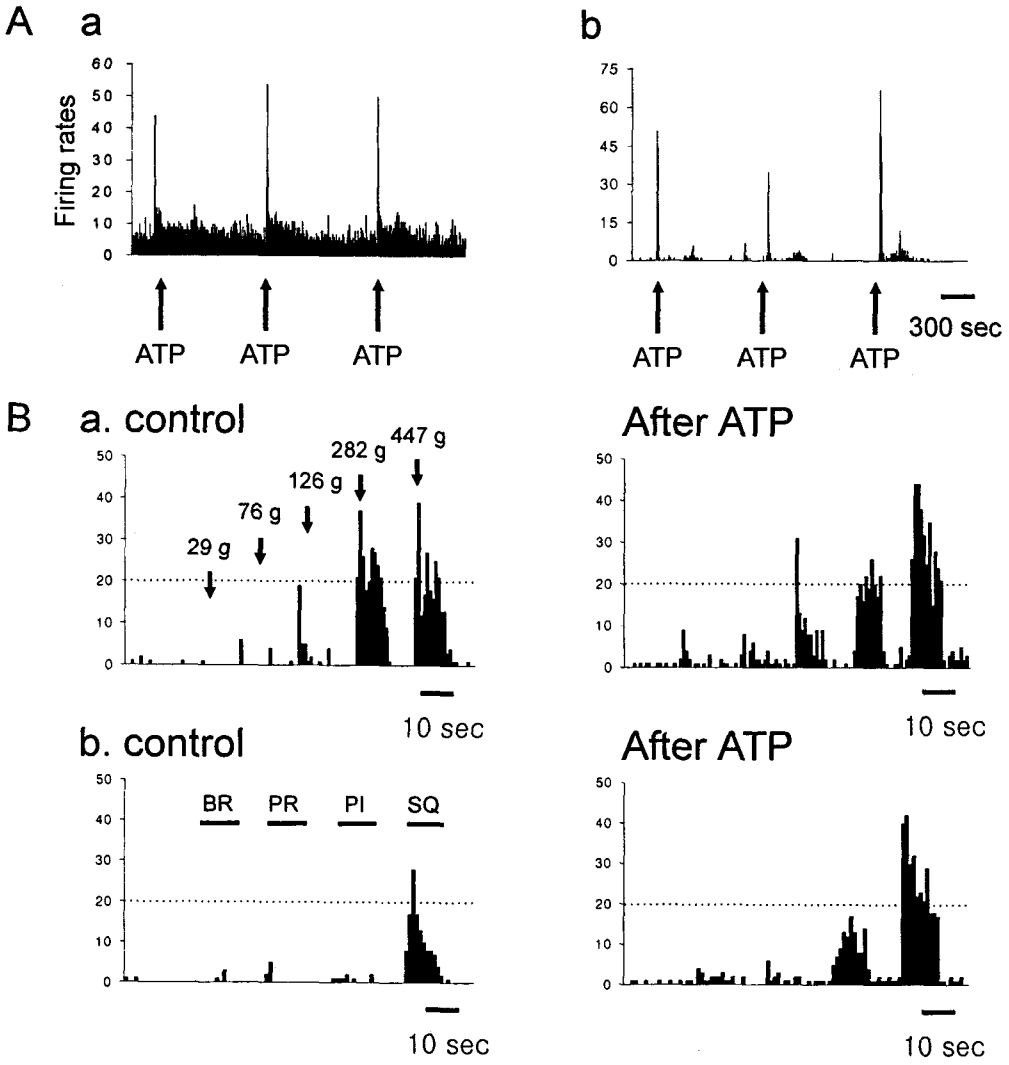

Fig. 3. Examples of responses of the dorsal horn neuron to ATP injection and peripheral noxious mechanical stimuli. (A) Intradermal application of ATP (20 μl, 5 mM) resulted in increase of the spontaneous activity (monophasic pattern). (B) Responses of a dorsal horn neuron to mechanical stimuli increased after ATP injection. Ba: von Frey filament, Bb: natural mechanical stimuli.

response was not observed in any cells. Fig. 3Aa showed the spontaneous excitation of single unit activity (initial response) at the same time with ATP injection. The responses evoked by intradermal ATP generally disappeared with short time courses within 400 sec. In case of ATP, repetitive application into the same site of receptive field is possible because ATP is a kind of endogenous substance and shows shorter time course for evoked-activity than that of exogenous formalin or capsaicin. When we conducted two or three times application of ATP, some cells show tachyphylaxis on the initial response (4/8). Fig. 3Ab showed both initial and slightly delayed-response around 200 sec, respectively, after initial response. In many cases (15/19), the responses of cells increased after ATP injection. The example is represented in Fig. 3B. This cell was high-threshold (HT) cell that responded only to near noxious and noxious mechanical stimuli. The threshold of this cell lowered from 126 g in control to 76 g one hour after ATP injection. Natural mechanical stimuli-evoked responses also increased. The firing rates responding to noxious squeeze exclusively in control had been changed consequently to that responding to stimulus of

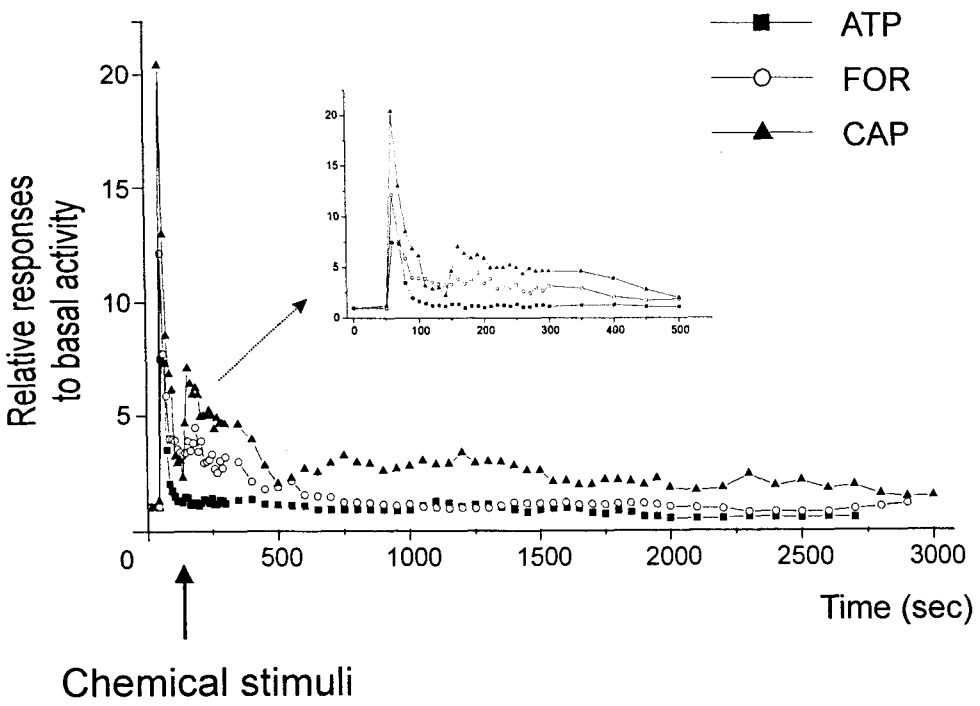

Fig. 4. Time course of responses of the dorsal horn neurons to peripheral noxious chemical stimuli (■, ATP, n=8; ○, formalin (FOR), n=11; ▲, capsaicin (CAP), n=11). Responses were expressed as a ratio of chemical-induced impulse rate to basal impulse rate. Chemicals were injected intradermally into receptive fields in the hind paw. Arrow (↑) indicates the time of injection (50 sec). Formalin- and capsaicin-induced responses were biphasic, while ATP-induced response was monophasic.

even lower strength as like near noxious pinch, which means hypersensitization.

The comparison of spontaneous activities evoked by intradermal injection of formalin, capsaicin, or ATP into receptive fields

Fig. 4 represented time-dependent changes of spontaneous firing rates evoked by intradermal injection of formalin, capsaicin, or ATP (20 μl) into receptive field of ipsilateral hind paw. The comparison was made in between the normalized mean number of each group and spontaneous activity (100%) of each group before chemical injection. The vertical axis represents the normalized mean number of spikes as a percent after each chemical injection. As showed in Fig. 4, application of formalin resulted in distinctive phases, namely, biphasic pattern with both initial increase of the spontaneous activities within 100 sec and delayed increase of the spontaneous activities 500 sec after formalin, which have similar time courses with that of known behavioral responses. In case of capsaicin, neuronal activities had also similar time courses with the results of formalin. However, the time course of initial response seemed to be shorter than that of formalin and other phase seemed to appear between initial phase and delayed phase, which considered as like triphasic pattern. In case of ATP, we only observed initial response, which disappeared away within 100 sec. Other cells (n=8), however, showed special features as Fig. 3Ab, which showed relatively rapid delayed response 200 sec after ATP, compared with that of capsaicin or formalin.

DISCUSSION

The goal of the present study was to determine whether the results of neuronal response to noxious chemical stimuli were consistent with those of behavioral study and whether each response was different according to types of chemical stimuli.

The responses of dorsal horn cell after formalin injection

We observed the changes of neural activities after intradermal formalin application into RF corresponding to the single unit activity of nociceptive dorsal horn neurons. Although the response of each was various in terms of the time courses of evoked responses, most cells showed monophasic (60%, n=6/10_{total}) or biphasic pattern (40%, n=4/10_{total}) after intradermal application of formalin. The biphasic patterns was reported by many behavioral studies (Dubuisson & Denis, 1977). Injection of formalin into a rat hindpaw produces a biphasic nociceptive response-licking, biting, shaking the injected paw-consisting of an early phase during the first 5min after formalin injection and a later phase starting after 15 min and lasting for 40~50 min (Chapman & Dickenson, 1993). These time courses of nociceptive responses to formalin in behavioral study are similar to those in our electrophysiological *in vivo* study. This consistence between behavioral study and single cell response means that biphasic behavioral response in formalin study may originate from activity of spinal cord dorsal horn cells. There is an experimental evidence (Dicken & Sullivan, 1987) showing the time course of subcutaneous formalin-induced activities of dorsal horn neurons in the rat-*in vivo* extracellular recording and their results are also consistent with ours.

Although the biphasic patterns in this study were similar to the results of other behavioral studies previously reported, the monophasic pattern evoked by formalin did not agree with both the results of the previous reported behavioral study and neural activity of the level of single cell *in vivo*. Monophasic patterns recorded in some cells seem to be considered as the following; 1) the biphasic patterns reported in the behavioral studies were the response of the level of organisms and animal, whereas the monophasic patterns we recorded were the cellular responses at the level of single unit in spinal cord. Therefore, the discrepancy of the mono- or biphasic patterns of the responses evoked by formalin can be occurred in terms of differences between the level of single cell and organism. 2) If the recording cell was interneuron, the neuronal activity evoked by formalin might also be changed because of the manifold feature of interneuron. In this study dorsal horn neurons recorded were not determined whether these were projection neurons sending their processes to supraspinal structure (e.g. thalamus) or interneurons. Thus, the activities of projection neurons after formalin injection need to be examined in order to distinct the response of projection neuron from that of interneuron.

Each phase of formalin-evoked responses might be explained by the followings. The initial phase after formalin might be evoked by direct excitation of the peripheral nociceptor, while the delayed phase seems to be related to central sensitization or peripheral sensitization. This delayed phase might be due to both direct C-fiber activation and central sensitization of spinal cord (Woolf, 1991; Dubner & Ruda, 1992; Tjolsen et al, 1992; Carli & Aloisi, 1993). The initial phase may be necessary to produce the central sensitization, which plays an important role in the appearance of the delayed phase (Shibata et al, 1989). It was also reported that inhibition for the initial phase did not induce both the delayed phase and central sensitization (Koppert et al, 1998).

In many cases, the responses of dorsal horn cells to innocuous and noxious mechanical stimuli increased after injection of formalin, which means allodynia and/or hyperalgesia. These patterns may be considered as the results of the central sensitization accompanied by the tissue injury and inflammation. In other cases, some cells showed the hypoalgesic response, which decreased the responsiveness to noxious stimuli after formalin injection. Neoh (1993) reported that the changes of RF were various according to injection site of chemical substance and the cells were hypoalgesic or hyperalgesic response. Also RF largely expanded and the sign of inflammation (rash, edema) was found at formalin injection site (Fig. 1).

The responses of dorsal horn cell after capsaicin injection

In present study, we investigated the effects of capsaicin on spontaneous activity of dorsal horn. Unlike formalin, neuronal activities evoked by capsaicin seemed to have other phase (rapid delayed phase) between initial phase and delayed phase, which considered as like triphasic pattern.

The mechanism of delayed response evoked by capsaicin, however, was not yet reported in detail, comparison with that of formalin. The possible mechanisms for delayed response might be considered as the following. 1) Capsaicin might act so repetitively and persistently at RF via peripheral C-fiber

activation that could result in 'wind-up' phenomenon. We implied that the rapid delayed-phase might have any correlation with 'wind-up'. This possibility could be supported by the clinical data that the flare or hyperalgesia caused by intradermal capsaicin in human progressively increased until a few minutes and then continuously declined. Actually, the rapid delayed phase in this study had the time courses between 150 and 350 sec. There is possibility as a driving force that the rapid delayed phase might induce the slow delayed phase. 2) Capsaicin might continuously excite the nociceptors around inflammation site because capsaicin is not easily hydrolyzed. This action of capsaicin might also show the prolonged effects on both primary afferent and nociceptive dorsal horn neuron. 3) Eventually, the delayed response might be associated with both peripheral mechanism and central mechanism, central sensitization (LaMotte et al, 1991, 1992). Central sensitization might be considered as the main mechanism of delayed response and this possibility has been supported by several reports (Woolf, 1991; Dubner & Ruda, 1992; Coderre et al, 1993).

Thus, the triphasic pattern after capsaicin injection might involve the initial response caused by direct excitation of nociceptor, the rapid delayed response by inflammation and selective C-fiber activation via vanilloid receptor, and the slow delayed response by inflammation and central sensitization. In the case of formalin application, we could not find any rapid delayed response.

The responses of dorsal horn cell after ATP injection

ATP is a kind of endogenous substance that can be released at tissue injury site or inflammation. ATP binds to P2X receptor in sensory neurons and excites nociceptor (Chen et al, 1995; Lewis et al, 1995). In present study, we implied that ATP might directly excite nociceptor via P2X receptors and then immediately diffuse or hydrolyze because the ATP-induced responses only showed monophasic pattern (8/8), which disappeared within 100 sec and then recovered basal level. Although RF was slightly expanded after ATP injection, there was not found any sign related to inflammation (Fig. 3).

From the above results, it is concluded that ATP directly stimulates peripheral nociceptors, capsaicin affects the peripheral C-polymodal nociceptors as well as induces neurogenic inflammation and central sensitization, and formalin stimulates peripheral nociceptor, and induces local inflammation and central sensitization. Probably the pain signals might be processed via different neural mechanisms in a dependent manner for kinds of the chemical stimuli.

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