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Effects of Steroidal and Non-steroidal Anti-inflammatory Drugs on Bradykinin-evoked Responses of Nociceptors from the Rat Temporomandibular Joint

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Abstract: Previous studies indicate that inflammatory processes of the temporomandibular joint (TMJ) may easily trigger pain. In this study, we tested the effects of two anti-inflammatory drugs on the response properties of C-polymodal nociceptors from the rat's TMJ, *in vitro*. Male Wistar rats weighing 250 to 350 g were used. C-polymodal units were identified based on conduction velocity (<2.0 m sec^{-1}) and responsiveness to thermal stimuli. Nociceptive responses were induced by injection of bradykinin (BK) into the TMJ area, followed immediately by the topical application of either flurbiprofen, dexamethasone, or physiological saline (as a control) to the receptive field. Both flurbiprofen and dexamethasone significantly reduced the BK-induced firing rate of nociceptive units. In addition, both drugs significantly reduced the BK-induced mechanical sensitization. However, flurbiprofen and dexamethasone worked in different time windows through clearly different mechanisms, suggesting that the concomitant use of these drugs could be even more effective than either drug alone.

Key words: Polymodal nociceptor, flurbiprofen, dexamethasone, TMJ-nerve preparation, rat

INTRODUCTION

The temporomandibular joint (TMJ) has unique features that render it easily susceptible to functional disorders, leading to inflammation and pain (Weiskopf, 1979). Despite spontaneous or provoked pain of varying degrees, TMJ disorder patients often suffer from pain-related secondary symptoms such as tension, anxiety, depression, and loss of appetite (Yap *et al*., 2002). Although the cause and effect relationship with some secondary symptoms may be questionable, TMJ disorders are frequently associated with a poor general health condition. Therefore, careful and efficient pain control therapy is of paramount importance for TMJ patients.

Disorders of the TMJ have been the object of numerous studies, both basic and clinical (Anderson *et al*., 1970; Boering, 1979; Rayne, 1987; McKay *et al*., 1992; Rantala *et al*., 2003). In recent years, Takeuchi *et al*. (2001, 2004). Takeuchi and Toda (2003) have reported on a novel *in vitro* model for the study of pain in the TMJ. This model allows for the investigation of response properties from individual nociceptive units in a strictly controlled environment. From their results so far, it can be gathered that polymodal nociceptors are predominant in the TMJ area (75.8% of the 33 nociceptive units recorded) and would thus be the receptor type predominantly involved in TMJ pain. Polymodal nociceptors, as defined by Kumazawa *et al*. (1977), are nociceptive units sensitive to mechanical, chemical and thermal stimuli. Hence, inflammation of the TMJ capsule may readily evoke pain sensation in the area.

Among the anti-inflammatory drugs, two main groups can be distinguished. Non-steroidal anti-inflammatory drugs, or NSAIDs and steroidal drugs. NSAIDs are the most common drugs used clinically for pain control. The mechanism of action of NSAIDs is based on the inhibition of the enzyme cyclo-oxigenase, which blocks the conversion of arachidonic acid into eucosanoid hormones that produce pain and sensitization to noxious stimuli (Vane, 1971; Vane and Botting, 1998). Steroidal drugs, on the other hand, work on the inhibition of gene expression triggered by inflammatory tissue reaction (Barnes, 1998). Steroids are widely used for the treatment of chronic inflammatory processes, such as arthritis and rheumatoid arthritis. In this study, we used the TMJ-nerve *in vitro* model to assess the effects of two representative anti-inflammatory drugs (NSAID, flurbiprofen and Steroidal, dexamethasone) on the response properties of single C-polymodal nociceptive units from the rat TMJ.

MATERIALS AND METHODS

The experimental procedures described here were in agreement with the Animal Care Standards of Nagasaki University and had the approval of its Animal Welfare

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Committee. The experiments were performed in Nagasaki, Japan, at the Laboratory of Integrative Sensory Physiology, Nagasaki University, from April to October, 2004.

Animal preparation: The *in vitro* TMJ-nerve preparation and the single fiber unit recording technique were used. Preparation and recording have been previously described in detail and validated (Takeuchi *et al*., 2001, 2004; Takeuchi and Toda, 2003). Briefly, male Wistar albino rat weighting about 250 to 350 g were deeply anesthetized with thiamylal sodium (80 mg kg^{-1}, i.p., Isozol®, Yoshitomi Pharmacy, Osaka, Japan) and the skin and muscles of the neck were locally anesthetized with lidocaine chlorate injections (1% Xylocaine®, Fujisawa Pharmacy, Osaka, Japan). After amputation of the neck, the skin of maxillofacial area was stripped. Then, the posterior area parts of the temporal and masseter muscles were detached to clearly expose the TMJ area including the capsule and retrodiscal tissues. The skull was divided at the central suture. The auriculo-temporal nerve was identified on the surface of the lateral pterygoid muscles, separate from the surrounding tissues, and sectioned at the foramen ovale to obtain as long a segment of it as possible. During surgery, TMJ and the auriculo-temporal nerve was kept in modified Krebs-Henseleit solution to avoid dehydration. For accuracy, binocular microscope (20×, Nikon-46207, Tokyo, Japan) was used.

The TMJ preparation was mounted in organ bath with Krebs-Henseleit solution, which was continuously perfused with a gas mixture (95% O_2, 5% CO_2) resulting in a pH around 7.4. The mandibular ramus was set in a vertical position and fixed on utility wax and dental cement. The auriculo-temporal nerve trunk was ligated with a cotton thread and passed through a hole (diameter 1 mm) into an adjacent recording chamber filled with paraffin oil.

Electrophysiological recordings and unit classification: To record single unit discharges from auriculo-temporal nerve, we used the microneurographic technique (Hagbarth and Vallbo, 1969). Unit discharge was recorded with a tungsten microelectrode (tip impedance: 12 MΩ, A-M Systems Carlsberg, USA) and amplified by DAM-80E (World Precision Instruments, Sarasota USA). Spikes were displayed on a personal computer through a CED 1401 interface (Cambridge, UK). Unit responses to various stimuli were analyzed the software Spike 2 (Version 2.01).

With a bipolar tungsten needle electrode (tip impedance: 2.0 mm), electrical stimulation was applied to the receptive field to estimate the conduction velocity was corrected to the value at the 37°C using the Q_{10} value reported by Paintal (1965). We further investigated units that had conduction velocity under 2.0 m sec^{-1} and showed response to heat 40 to 50°C heat stimulation, which were thus categorized as C-polymodal nociceptive units (Beitel and Dubner, 1976; Koltzenburg *et al*., 1997).

Test drugs: Excitation and sensitization of nociceptors was led by BK (10^{-4}M, 0.1 mL), witch was injected into the TMJ tissues through a 27-gauge needle. Immediately after BK injections, the test drugs were applied to the respective receptive fields over the TMJ area. We used followings as test drugs: physiological saline as a control; flurbiprofen (0.01% in glycerin, Ropion®, Kaken, Tokyo, Japan,) as a non-steroidal anti-inflammatory drug; and dexamethasone (0.03% in Krebs-Henseleit solution, Wako, Osaka, Japan) as a steroidal anti-inflammatory drug. These drugs were applied topically (each total volume of 1.0 mL) through a plastic cylinder (inner diameter: 3 mm) closely placed over the receptive field to prevent leakage to surround tissues.

Mechanical stimulation: Mechanical stimulation was applied to C-polymodal units 3 times over a period of 5 min. A von Frey-type apparatus was used, consisting of a rigid metal needle (tip diameter: 0.4 mm) fixed to a calibrated strain gauge and its amplifier (TBM 4, World Precision Instruments Inc., Sarasota, FL, USA) (Takeuchi *et al*., 2001). The signal was also fed into a personal computer through the CED 1401 interface. The tested units were either normal (non-treatment), BK-and BK and test drug-treated.

Data analysis: All data are presented as mean±SEM. Data from different experimental groups was compared by ANOVA followed by Fisher's PLSD test (Statview, SAS Institute, Cary, NC, USA). A $p<0.05$ was considered significant.

RESULTS

BK evoked firing frequency: A total of 35 C-polymodal units were recorded from the rat TMJ. Five units received BK stimulation only; the remaining units were treated with saline solution, flurbiprofen, or dexamethasone (n = 10 each). Figure 1 shows the time course of changes in the BK-induced firing rate. Units treated BK only showed spontaneous firing at a rate of about 2 Hz during the first 30 sec, the firing frequency then lowered about 15% at 60 to 90 sec and remained stable for the remainder of the observation period (300 sec). Units treated with BK and Saline solution showed strictly the same behavior as BK only units, with no statistically significant differences throughout the experimental

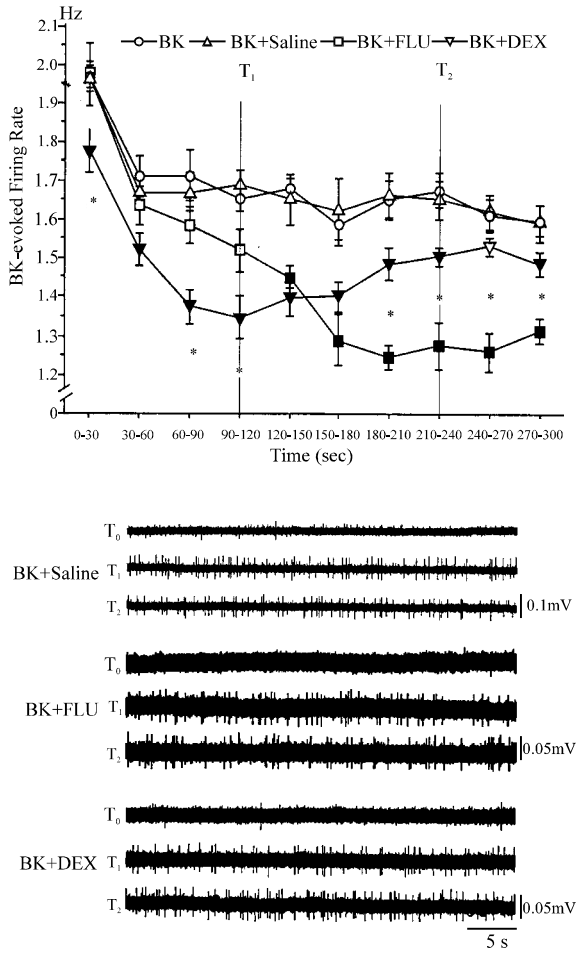

Fig. 1: Time course of changes in the BK-evoked firing rate and example of spontaneous activity following anti-inflammatory drug. In each group, n = 10 units. T_0: Unit activity recorded 60 to 30 seconds before the application of bradykinin. T_1: At 90 to 120 sec, units treated with BK and dexamethasone (DEX) showed peak reduction of firing frequency. T_2: At 210 to 240 sec, the reduction of the discharge to flurbiprofen (FLU) treated units remained significantly lower for the remainder of the experimental period. Closed symbols indicate statistically significant difference compared to BK and Saline group; asterisks indicate significant difference between Flurbiprofen (BK+NSAID) and Dexamethasone (BK+Steroid) groups ($p<0.05$ in ANOVA followed by Fisher's PLSD). Values are mean±SEM. B: Firing pattern of raw data at T_1 and T_2 in each group

period. When compared with BK and saline units, units treated with BK and flurbiprofen showed reduced firing frequency at 60 to 90 sec and reached statistical

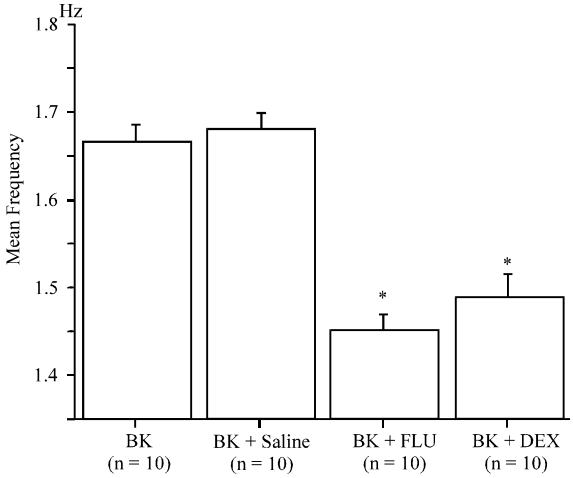

Fig. 2: Mean firing frequencies of C-polymodal nociceptors over the 5 min observation period. Flurbiprofen (BK+FLU) and Dexamethasone (BK+DEX) had an overall similar effect in suppressing BK-evoked responses. Asterisks indicate statistically significant differences versus BK and saline group ($p<0.05$ in ANOVA followed by Fisher's PLSD). Values are means±SEM

significance at 120 to 150 sec. The firing frequency of flurbiprofen treated units remained significantly lower for the remainder of the experimental period. The peak reduction in BK-induced firing frequency was observed at 180 to 270 sec, about 25% lower than BK and saline units at the corresponding time period. Units treated with dexamethasone, on the other hand, showed significantly lower firing frequency already during the first 30 sec and remained significantly lower than BK and saline units for most of the 300 sec observation period. In dexamethasone treated units, the peak reduction in BK-induced firing frequency (about 17% lower than BK and saline units) was observed at 60 to 120 sec. Only small differences were observed after 240 sec, although still significant at 270 to 300 sec. When flurbiprofen- and dexamethasone-treated units were compared, flurbiprofen-treated units showed significantly lower firing frequency at 180 to 300 sec, while dexamethasone-treated units showed significantly lower firing frequency at 0 to 30 sec and at 60 to 120 sec.

During the whole 5 min (300 sec) observation period, the mean firing frequency of units treated BK and flurbiprofen, and BK and dexamethasone was significantly lower than those treated BK and saline. Statistically significant differences were not found between BK only and saline, or between flurbiprofen- and dexamethasone-treated units (Fig. 2).

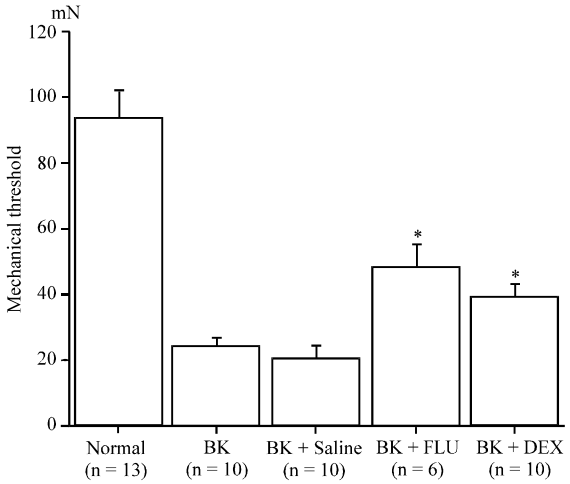

Fig. 3: Mechanical thresholds from different experimental groups. All BK-sensitized groups showed significantly lower mechanical thresholds than the normal (untreated) group. Asterisks indicate statistically significant differences versus BK and saline group ($p<0.05$ in ANOVA followed by Fisher's PLSD). Values are means±SEM

Mechanical threshold: Mechanical thresholds were recorded from 49 units. Each unit was tested 3 times during a period of 5 min. Figure 3 shows the mean mechanical thresholds of normal (untreated, n = 13), BK (n = 10), BK and saline (n = 10), BK and flurbiprofen (n = 6) and BK and dexamethasone (n = 10). The mechanical thresholds of all BK-treated groups were significantly lower than that of the normal group. However, BK and flurbiprofen and BK and dexamethasone groups showed significantly higher mechanical thresholds than the BK and saline group. Statistically significant differences were not found between BK only and BK and saline groups, or between BK and flurbiprofen and BK and dexamethasone groups.

DISCUSSION

BK has been regarded as not merely a mediator of inflammation, pain, and hyperalgesia, but as the most potent endogenous algogenic substance known (Armstrong *et al*., 1953; Kantor *et al*., 1967; Keele, 1970; Rang *et al*., 1991; Dray and Perkins, 1993; Dray, 1995; Calixto *et al*., 2000). In the normal tissues, BK is made *de novo* from kininogen precursors under pathophysiological stimuli such as tissue injury, hypoxia, and low pH (Dray and Perkins, 1993; Dray, 1995; Calixto *et al*., 2000). BK acts locally and produces a great variety of tissue responses including those that characterize inflammatory processes. The effects of BK on TMJ tissues were of particular interest here, as numerous studies link BK with the etiology of pain from inflammatory and rheumatoid diseases (Dray and Perkins, 1993).

It is well known that subcutaneous injection of BK readily evokes pain sensation in humans (Kantor *et al*., 1967). BK affects nociceptive neurons through specific BK receptors. So far, there is accumulated evidence for the existence of at least two types of BK receptors, named B1 and B2 (Calixto *et al*., 2000, 2004). The physiological role of the B1 receptor remains unclear, as this receptor is not expressed to a significant extent in normal tissues. The expression of B1 receptors is stimulated by infection and inflammation and increases over a period of hours (Hall, 1997; Calixto *et al*., 2004). The B2 receptor, on the other hand, mediates most actions of BK, including activation of nociceptors and production of pain (Banik *et al*., 2001; Calixto *et al*., 2004). Likewise, the effects of BK observed in this study are likely a result of the activation of B2 receptors in TMJ tissues.

In this study, both anti-inflammatory drugs decreased the BK-induced firing rate of C-polymodal nociceptive units in the rat TMJ. However, steroidal and non-steroidal drugs acted differently over time. Dexamethasone was most effective during the fist 150 sec, while flurbiprofen was effective only 120 sec after application. This difference may be explained by the mechanism of action of BK on nociceptive neurons. The effects of BK itself are short-lived, as BK is rapidly metabolized by proteolytic enzymes that abound in most bodily tissues (Couture *et al*., 2001). However, the activation of B2 receptors stimulates a cascade of cellular and vascular events that includes the stimulated production of eucosanoid hormones from many tissues (Calixto *et al*., 2000). Thus, other chemical inflammatory mediators such as prostaglandins, cytokines, histamine, and 5-HT, which are secondarily produced after the stimulation of B2 receptors, sustain the excitation and sensitization of nociceptors. Therefore, flurbiprofen, whose effect is attributed to the inhibition of arachidonic acid sub-products, would work later that dexamethasone, which has been suggested to act directly on the cell membrane of nociceptive neurons (Johansson *et al*., 1990). Dexamethasone, however, would not be able to suppress eucosanoid release sufficiently (Werner *et al*., 2002) and thus attenuate the excitation of nociceptors on the same time window where flurbiprofen was most effective. Nevertheless, when the entire 5 min observation period was taken in account, both anti-inflammatory drugs had a similar effect in reducing nociceptors' firing frequency. In the long run, clinical studies suggest NSAIDs to produce greater initial analgesia by inhibition of eucosanoid formation and steroids to provide better

suppression of swelling and less loss of function (Troullos *et al*., 1990).

Injection of BK into TMJ tissues dramatically reduced the mechanical threshold of C-polymodal nociceptors. This result is in line with those of others where BK application effectively reduced the mechanical threshold of nociceptors in deep tissues, but not in the skin (Liang *et al*., 2001). In fact, one of the most prominent characteristics of TMJ pain is its exacerbation during mandibular movements. Both anti-inflammatory drugs significantly raised the nociceptors' mechanical threshold, but not to normal values. Therefore, the TMJ may remain somewhat sensitive to mechanical stimulation despite anti-inflammatory treatment. This result may in part explain why acute TMJ pain must often be treated with local anesthetic blocks to completely subside.

In conclusion, both non-steroid and steroid drugs showed significant analgesic effect on C-polymodal nociceptors in the TMJ. The two drugs studied here worked through clearly distinct mechanisms, suggesting that concomitant use of both drugs could be of even greater clinical value than either drug alone (Potter, 2005).

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