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Rapid communication

Interactions of bradykinin and norepinephrine on rat cutaneous nociceptors in both normal and inflamed conditions in vitro

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Abstract

Many inflammatory chemical mediators excite or sensitize nociceptors, which had led some researchers to believe that they may interact with each other to maintain a persistent painful state. We examined how the excitatory mediators norepinephrine (NE) and bradykinin (BK) interact, using single fiber recordings from cutaneous nociceptors. We observed that NE augmented the BK-induced response in both control and adjuvant-inflamed rats in a way different from NE-induced excitation in inflamed animals only. BK also tended to augment the NE-induced response (examined only in inflamed rats). Our results provide the first evidence that BK and NE synergistically interact on nociceptors.

Keywords: Rat; Norepinephrine; Bradykinin; Inflammation; Cutaneous nociceptor

During an inflammatory event, primary afferent fibers are exposed to various chemical substances, such as prostaglandins, bradykinin (BK), ATP, and protons. Electrophysiological findings have shown that many of these substances facilitate nociceptor activities (Mizumura and Kumazawa, 1996; Mizumura, 1998 for review). It has been suggested that by interacting with each other on the nociceptor, these substances have a concerted and amplified effect on the chemosensitive nociceptive endings (Steen et al., 1995). Of these substances, BK is the most potent in exciting nociceptors and in sensitizing them to thermal and mechanical stimulation (Mizumura and Kumazawa, 1996; Koda and Mizumura, 2002). Therefore, examining possible interactions between BK and other mediators would be useful in understanding the factors contributing to inflammatory hyperalgesia.

The contribution of sympathetic nerve activity to some neuropathic pain conditions has been widely accepted, and thus the mechanism of interactions between sympathetic and afferent systems has been intensively studied (Baron, 2000 for review). Although the contribution of such interaction to inflammatory hyperalgesia has also been suggested at the behavioral level (Baik et al., 2003; Raja, 1995), the mechanism for this at the nociceptor level remains to be elucidated. Our previous studies have shown that a sub-population of cutaneous C-fibers exhibited evoked responses to exogenous norepinephrine (NE) during persistent inflammation in both in vivo (Sato et al., 1993) and in vitro (Banik et al., 2001b) recordings. In this study we examined whether there was a cooperative interaction between bradykinin, one of the most potent hyperalgesic inflammatory mediators, and NE in control as well as in inflamed conditions.

A preliminary report has appeared in abstract form (Sato et al., 1998).

Experiments were carried out on male Lewis rats, 180–200 g (Charles Rivers, Japan), because the NE sensitivity in inflamed condition was higher in this strain (Banik et al., 2001b). The experimental procedures for development of the polyarthritic rat model used have been described elsewhere (Banik et al., 2002). Briefly, 0.1 ml complete Freund's adjuvant (CFA), a suspension of heat killed *Mycobacterium butyricum* (Difco, Detroit, USA) in mineral oil (6 mg/ml), was injected into the distal third of the tail. About two weeks after inoculation of CFA, all rats developed inflammation in many places including many joints

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and skin. Rats receiving no injection of CFA were used as controls. To minimize the discomfort of animals, rats that developed sings of inflammation were isolated into separate cages. The number of inflamed animals used was kept to a minimum. The experimental procedures for in vitro single nerve fiber recording (Reeh, 1986) were described in our previous report (Banik et al., 2001a). The saphenous nerve was subcutaneously dissected with its innervated territory on the hairy hindpaw skin until the nerve and skin could be removed. The skin was placed 'epidermal side down' in the in vitro perfusion chamber and superfused with a modified Krebs–Hensleit solution (in mM: 110.9 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2SO_4, 24.4 $NaHCO_3$ and 20 Glucose), which was saturated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at a temperature of $34 \pm 0.5\,^{\circ}C$. Single unit activities were recorded from the saphenous nerve by conventional dissection and amplification.

All chemicals were dissolved in Krebs solution and prewarmed at 34 °C before being applied to the stimulation chamber (receptive field of a C-fiber receptor). 10^{-6} M BK often failed to excite C-fiber receptors of control animals, as reported previously (Banik et al., 2001a), and we applied 5–10 times higher concentrations of BK in these cases. To compare the effect of NE on BK response, one group of C-fibers received BK for 1 min up to six times at 10 min interval (control); the same protocol was used in the other group except that 10^{-5} M NE was applied for 5 min before and during the fourth serial application of BK.

In another group of units of inflamed rats, the effects of BK on NE-induced excitation were studied. All units received 10^{-5} M NE twice to their receptive field for 5 min at 30-min intervals. In these cases receptive fields were not challenged by BK before the first NE application. About half of them (BK-treated group) received 10^{-6} M BK for 5 min just before the second application of NE, while the rest did not (control group).

To count the total number of impulses induced in response to BK or NE application, the impulse number, if any, during 1-min control period was multiplied by 5 (10 for NE in the experiment of BK effect on NE) and then subtracted from the count for 5 min (10 min for NE in the experiment of BK effect on NE) after BK or NE application. Values are expressed as relative increases or decreases of the total evoked spikes from the previous response. The data was presented as mean ± S.E. The Wilcoxon signed rank test was used to analyze the paired comparison, and Mann–Whitney test was used for un-paired comparison. A two-tailed value of $P < 0.05$ was taken to be statistically significant. BK was obtained from Peptide Institute Inc. (Minoh-shi, Osaka, Japan) and NE from Sigma (St. Louis, MO, USA).

We carried out all experiments with the approval of the Animal Care Committee, Nagoya University.

In this study 44 mechanoheat-sensitive receptor units were recorded. The conduction velocity of these units ranged 0.1–3.5 m/s, with the conduction velocity of the great majority of fibers in the C-fiber range.

In 19 control and 15 inflamed units, 10^{-6} M BK (5 or 10×10^{-6} M in control rats) was applied for 1 min at 10-min intervals up to six applications. During repeated 10^{-6} M BK applications C-fibers were excited by the second successive application with a considerably decreased discharge, and then adapted within 3–6 applications to a stable state. The responses to the third and fourth applications of BK to C-fibers from control Lewis rats ($n = 7$) were 22 ± 9.7 impulses and 21.5 ± 8.4 impulses, respectively, and to those from inflamed Lewis rats ($n = 7$) were 54.0 ± 14.0 impulses and 59.9 ± 15.8 impulses. Differences between responses to the third and fourth BK applications were not statistically significant in either group (Fig. 1B and C left). As the concentration of BK used was different between the two groups, the magnitude of response was not compared between them.

As shown in Fig. 1A, NE facilitated the BK response in C-fibers recorded in control and inflamed rats. Such augmentation was observed in 7 of 12 receptors (58%) from control rats and in 6 of 8 receptors (75%) from inflamed rats. The incidence was thus not significantly different between the control and inflamed rats ($P > 0.1$, χ^2 test). On average, NE significantly augmented the BK-induced response, from 34.3 ± 10.9 impulses to 69.2 ± 23.1 impulses in control rats (right graph in Fig. 1B, $n = 12$, $P < 0.05$) and from 33.5 ± 17.1 impulses to 57.1 ± 17.9 impulses in inflamed rats (right graph in Fig. 1C, $P < 0.05$, Wilcoxon signed rank test). The magnitude of facilitation was not different between groups ($P > 0.8$, Mann–Whitney test). The augmenting effect was not observed in the response 10-min later (fifth serial application, see Fig. 1A).

In control rats, NE did not evoke any impulses when applied for the first time before BK application. This is consistent with the previous observations (Shea and Perl, 1985; Barasi and Lynn, 1986). However, NE applied after three BK applications often produced low frequency intermittent discharges (example in Fig. 1A) in control rats, and such activities by NE after BK applications were seen in 9 of 12 (75%) C-fiber receptors. The magnitude of response ranged from 3 to 53 impulses (average 18.7 ± 6.5 impulses during 5-min application period).

As we reported before (Banik et al., 2001b) the same concentration of NE produced excitation in inflamed C-fibers in preparations which was not previously challenged by BK. We expected that this NE-sensitivity of inflamed C-fibers would be enhanced after BK applications. However, this was not the case as only four out of eight C-fibers (50%) from inflamed rats showed NE-sensitivity after BK applications, with a response magnitude ranging 3–67 impulses (average 13.9 ± 8.5 impulses). A comparison of these data with NE-sensitivity in preparations that were not challenged with BK (data from Banik et al., 2001b; 17 out of 26 units were NE-responsive) revealed no significant differences.

Since the appearance of NE-sensitivity was clearly observed after BK application even in control preparations, we next addressed the question of whether BK has any effect on NE-sensitivity. In inflamed rats, our previous data

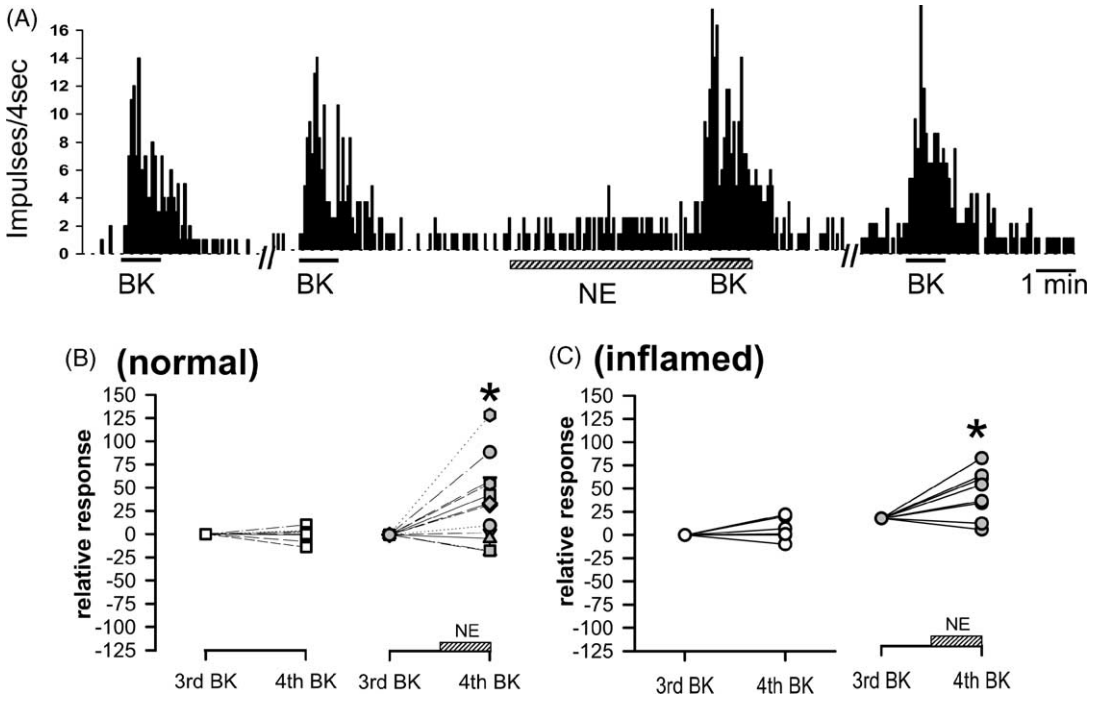

Fig. 1. Augmenting effect of norepinephrine (NE) on responses to bradykinin (BK) of cutaneous receptor unit innervating the rat hindpaw skin in control and adjuvant inflamed Lewis rats. (A) Continuous recording was taken from a single mechano-heat sensitive receptor unit of an inflamed Lewis rat during repeated BK (10^{-6} M) applications at 10-min intervals. (//) shows that the recording was interrupted for several min at this point. The first NE application before any BK application was not shown because there was no response, and the first response to BK was omitted because of space limitation. NE (10^{-5} M) was applied from 5-min before and during the fourth serial application of BK. BK application period is marked with the solid line and NE with obliquely hatched column. Note that the NE treatment induced a small increase in discharge rate after BK application, although NE failed to excite this receptor when applied before any BK application (not shown). (B) and (C): Responses to third and fourth application of BK are displayed. Ordinate: relative increases or decreases of the total evoked spikes (total spikes generated during 5 min after onset of BK-superfusion) before and after NE application. The NE treatment significantly augmented the BK-induced response ($P < 0.05$, Wilcoxon singed rank test) when compared with the control previous response (third response in the absence of NE).

(Banik et al., 2001b) showed that the response to 10^{-5} M NE could be reproduced in two applications when applied at 30-min intervals, and that the first NE response is a little larger than the second. In the present series, response to the second NE application was significantly smaller than that to the first, although the difference was small (Fig. 2B). Using the same protocol, four mechanoheat-sensitive units from inflamed rats were treated with 10^{-6} M BK in the last 5 min before the second NE application. All units responded to 10^{-6} M BK and in all but one of them the second NE response was greater. A specimen record of a BK responsive unit is shown in Fig. 2A. It must be mentioned that the discharge pattern responding to NE after BK application was similar to the preceding response to BK (Fig. 2A). On average, NE-induced discharges increased from 30.5 ± 24.3 impulses to 162.0 ± 66.3 impulses ($n = 4$) after BK application. Although a small number of units examined did not allow us to statistically analyze this data, there was a tendency that BK augmented the following response to NE.

The present experiment showed that NE facilitated the following BK response of C-fiber receptors in control and inflamed rats. The facilitating effect of NE reported in this paper is different from NE-induced excitation in that NE excited C-fiber receptors in naïve preparations only from inflamed animals (Sato et al., 1993). It is also different from the NE effect on the heat response. NE facilitated the heat response in inflamed animals but suppressed it in control animals (Yajima et al., 2000). These observations might suggest that BK produced a condition similar to inflammation, possibly through production of prostaglandins (Lembeck et al., 1976) that sensitize nociceptors to bradykinin (Mizumura et al., 1991), heat (Mizumura et al., 1993) and mechanical stimulation (Koda and Mizumura, 2002). It is not known whether BK modulates the receptor for NE, possibly the α2 adrenoreceptor (Sato and Perl, 1991). Prostaglandins have been implicated also in NE-induced sensitization to mechanical stimulation (Gold et al., 1994); thus, the NE-induced sensitization of bradykinin response reported here might also be mediated by prostaglandins.

NE-induced excitation tended to be facilitated after BK application by treating BK before second NE application, and the response to NE after BK applications looked almost similar to the previous BK-response, suggesting that the response to residual BK was facilitated and had become apparent. One of the proposed mechanisms for BK-evoked excitatory action of nociceptors is its strong sensitizing effect on the heat response (Kumazawa et al., 1991) leading nociceptors to be excited by the temperature of the bath

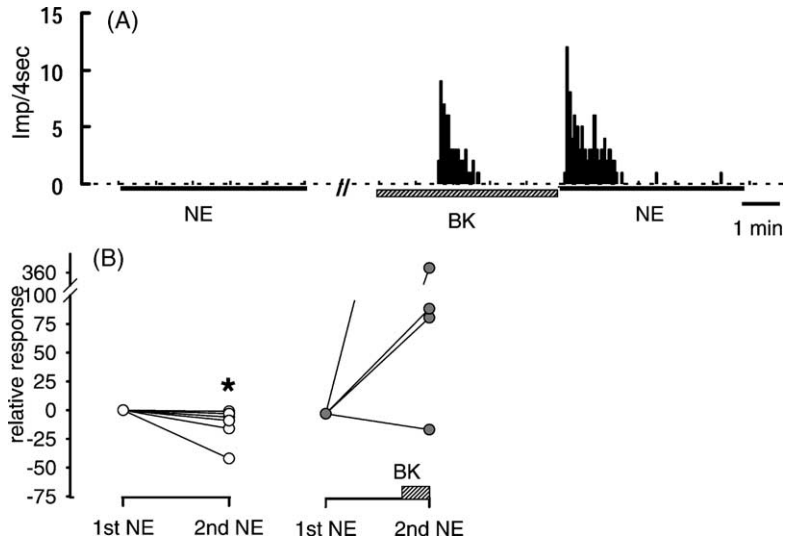

Fig. 2. Augmenting effect of bradykinin (BK) on responses to norepinephrine (NE) of cutaneous receptor unit innervating the rat hindpaw skin after adjuvant arthritis. (A) Continuous recording was taken from a single mechano-heat sensitive receptor unit during two consecutive applications of NE (10^{-5} M) at a 30-min interval. BK (10^{-6} M) was applied from 5-min before the second NE application. (//) shows that the recording was interrupted at this point. BK application period is marked with obliquely hatched column and NE with solid line. The bradykinin response appeared with a long latency and showed desensitization, this has been already reported (for review see Mizumura and Kumazawa, 1996). Note that the response pattern to second NE was quite similar to the previous BK response. (B) Responses to first and second application of NE are displayed. The numerical data represent relative increases or decreases of the total evoked spikes (total spikes generated during 5 min of application and 5 min after the end of NE-superfusion) before and after BK application. The second NE response in the absence of BK (left graph) was significantly smaller than the first ($P < 0.05$, Wilcoxon singed rank test), and that in the presence of BK tended to be larger but statistically not different from the control first response (right graph).

solution or of the body in vivo (Liang et al., 2001). This hypothesis is agreeable with a recent publication that BK, through protein kinase C activation, causes a shift of the heat-activation threshold temperature of a heat-sensitive ion channel (vanilloid receptor 1 or TRPV1) down to skin temperature (Sugiura et al., 2002). In rat cutaneous nociceptors from the same inflammation model as the present, NE has been shown to reduce the threshold of the heat response or to increase the magnitude of the heat response (Yajima et al., 2000). Taken together, this evidence suggests the possibility that NE augments the residual BK-evoked heat sensitization, resulting in apparent excitation.

Recently Seyedi et al. (1999) reported that the stimulation of cardiac sensory C-fibers by BK or capsaicin increased NE release from adrenergic terminals. In addition, exogenous BK enhanced NE exocytosis from cardiac sympathetic nerve endings of Guinea pig by 7% to 35% (Seyedi et al., 1997). In view of these results, one may expect that exogenous BK could augment local NE concentration of the tissue, which enhances the magnitude of its response. This might be another mechanism for NE-induced excitation after bradykinin application.

NE-induced excitation of the nociceptors observed in most studies was so small (Sato and Perl, 1991; Sato et al., 1993) that it may be doubted whether such a low frequency of discharges could contribute to pain, since similar activity did not evoke frank pain in humans (Ochoa and Torebjork, 1989). Our previous studies and the current one indicate a novel action of NE in that although the excitation by NE alone is negligible, its sensitizing effect upon BK could have a greater role in the generation of pain. A similar action was observed in the case of prostaglandin E2 and I2, which are potent agents in sensitizing BK and heat responses of visceral nociceptors although they do not excite nociceptors by themselves (Mizumura et al., 1987, 1991, 1993). Steen et al. (1995, 1996) reported that the effect of inflammatory soup (mixture of inflammatory mediators including BK) on cutaneous nociceptors was potentiated by lowered pH, and the present data provides additional evidence to our previous data (Mizumura et al., 1987) that an individual inflammatory mediator interacts with the action of BK. In conclusion, the present data together with our previous data (Sato et al., 1993; Banik et al., 2001b; Yajima et al., 2000) indicate a substantial involvement of NE in the aftermath of inflammatory events.

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