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Norepinephrine reduces heat responses of cutaneous C-fiber nociceptors in Sprague–Dawley rats in vitro

Jun Sato*, Hiroki Yajima, Ratan Kumar Banik[1], Takao Kumazawa[2], Kazue Mizumura

Department of Neural Regulation, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan

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Abstract

Nociceptors are excited or sensitized by many inflammatory mediators as well as by elevation of tissue temperature. We have shown that there is a facilitatory synergistic interaction between norepinephrine (NE) and bradykinin (BK) on cutaneous C-fiber nociceptors in normal Lewis rats. These interactions may play an important role in the mechanism of sympathetically maintained pain. In the present experiment, using skin-saphenous nerve in vitro preparations, we tested the effect of NE on the activity of nociceptive fibers and their response to heat in normal Sprague–Dawley rats. For comparison with the previous data on Lewis rats, we also examined the effect of NE on BK response. NE (10^{-5} or 10^{-6} M) did not excite nociceptive fibers before repeated heat stimuli or BK superfusion (10^{-5} or 10^{-6} M) to the receptive field. In contrast, after a few applications of heat or BK, NE excited 20–43% of nociceptive fibers to similar magnitudes. We also found that NE sensitized subsequent BK responses, but somewhat unexpectedly that it suppressed subsequent heat responses. This occurred regardless of the presence or absence of NE-induced excitation. These results suggest different mechanisms of NE modification to the BK and heat responses of cutaneous C-fiber nociceptors.

Keywords: Sprague–Dawley rat; Norepinephrine; Bradykinin; Heat; Sensitization; Desensitization; In vitro; Cutaneous C-fiber nociceptor

Studies have shown that sympathetic adrenergic activities can excite cutaneous nociceptors under inflamed but not normal conditions, suggesting that interaction between sympathetic nerve activity and inflammatory mediators plays an important role in inflammatory pain states [3,4,6,16,20]. A recent study by our group [2] showed that NE augmented the subsequent bradykinin (BK) responses of cutaneous nociceptors in both normal and adjuvant-induced arthritis Lewis rats. Additionally, BK augmented the following NE response in the inflamed animals. These results suggest facilitatory interactions between BK and NE on cutaneous nociceptors. An interesting question is whether the heat response is similarly influenced by sympathetic activities. We attempted to clarify the effect of sympathetic activities on the nociceptor response to heat in normal Sprague–Dawley rats, which are often used in the study of neuropathic and inflammatory pain. For comparison with the previous data on Lewis rats, we first examined the effect of NE on the BK response. A preliminary report has appeared in abstract form [18].

All the experiments in the present study received approval from the Animal Care Committee of Nagoya University. The experimental procedures are similar to those reported before [2,3] except that Sprague–Dawley were used instead of Lewis rats in the present study, even though NE sensitivity in these rats is reportedly lower than in Lewis rats [3]. Briefly, single C-fiber polymodal receptor (CPR) activities were recorded from the saphenous nerve using a skin-nerve in vitro preparation [15] excised from deeply anesthetized 26 male rats (pentobarbital 55 mg/kg, i.p.). The isolated organ was mounted with its corium side up in an organ bath and kept under laminar superfusion at a rate of 14 ml/min

* Corresponding author. Tel.: +81 52 789 3862; fax: +81 52 789 3889.
E-mail address: jun@riem.nagoya-u.ac.jp (J. Sato).
[1] Present address: Department of Anesthesia, University of Iowa School of Medicine, Iowa City, IA 52242, USA.
[2] Present address: Department of Algesiology, Aichi Medical University, Nagakute, Aichi 480-1195, Japan.

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with a modified Krebs 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 mM glucose), which was saturated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at a temperature of 34 ± 0.5 °C. Single CPR activity was analyzed on a computer with a SPIKE/SPIDI software package [5]. A small metal ring was placed over the receptive field (ring chamber) to isolate the receptive field from the superfusate. Chemical stimulation was carried out by superfusing the ring chamber separately (2.6 ml/min, 34 °C) with drug solutions for 1 min (10^{-6} or 10^{-5} M BK) or 5 min (10^{-6} or 10^{-5} M NE) using a push-pull pump. At the end of the drug superfusion, the receptive field was rinsed with warmed (34 °C) Krebs solution. In the control experiments, Krebs solution was superfused instead of the drugs. BK was obtained from Peptide Institute Inc. (Osaka, Japan) and NE from Sigma (St. Louis, USA). Stock solutions (10^{-3} M) of these drugs used were kept frozen (−80 °C) and were diluted to the relevant concentration in the Krebs solution on the day of use. For heat stimulation, solution in the ring chamber was removed and the receptive field was heated at 10-min intervals by a feedback controlled radiation stimulator (DPS 701, DIA MEDICAL, Japan) at the corium side. The skin temperature was monitored and raised from 34 °C at a speed of 0.65 °C/s. To avoid any possible damage of the receptive field, a "cut-off temperature" was introduced. To establish this cut-off temperature, the temperature was raised in the first heat stimulation until impulses were evoked at 5 Hz. The cut-off temperature thus established was then applied in the subsequent stimuli (10-min intervals) for a given unit.

The magnitude of the CPR response to the BK or heat stimulation was determined by counting the total number of impulses evoked. Since some CPRs had spontaneous discharges and/or after-discharges outlasting the stimulation period, the net total number of impulses induced from the initiation of the BK or heat stimulation to cessation of the response (i.e., total response period) was calculated according the formula: {(mean discharge rate during the total response period) − (mean discharge rate during the 30-s preceding control period)} × (total response period). In the series of heat experiments, the response threshold temperature was defined as the temperature that evoked the first spike by the heat stimulation. When a unit had spontaneous discharges, the threshold was determined as the temperature at which the instantaneous frequency of two successive spikes exceeded the mean instantaneous frequency during the 30-s control period preceding the heat stimulus by its standard deviation.

The magnitude of the NE responses of CPRs was determined by counting the net total number of impulses evoked during the 5 min after onset of the NE superfusion. For this purpose, the impulse number, if any, during the 30-s prestimulus control period was multiplied by 10 and subtracted from the total number of evoked impulses. NE-induced CPR discharges were weak and occurred at low frequencies, as has been reported previously [3,19,20], which led us to define "response to NE" as at least 10 impulses during the 5-min NE application.

Data are shown as mean ± standard error of mean (S.E.M.). Statistical analyses were performed using nonparametric Wilcoxon signed rank test (Wilcoxon test) and unpaired *t*-test, as appropriate. A difference was considered significant at the $P < 0.05$ level.

Forty-seven CPR units were investigated that had a conduction velocity ranging from 0.27 to 0.79 m/s. Seven units (15%) showed spontaneous discharges ranging from 2 to 7 impulses/30 s before any stimulation other than the search stimulus. No significant difference of the conduction velocity was observed between CPRs with or without spontaneous discharges ($P > 0.05$, unpaired *t*-test). The interactions between NE and BK or heat stimulation on the activity of nociceptors were studied in different groups of CPRs.

At a concentration of 10^{-6} M BK, only 3 of 12 CPR units (25%) responded. When the concentration was increased to 10^{-5} M BK, all the remaining 9 units responded, as did another 8 units tested first at this concentration. BK was applied 6 times at a concentration of 10^{-6} M in the 3 units that responded, and 10^{-5} M in the others (17 units). The excitement of the CPRs produced by the second application of BK resulted in considerably decreased discharges compared to the first application (tachyphylaxis), and then remained at this level. This response characteristic of polymodal nociceptors was consistent with our previous observations [1,12]. After the third application of BK at concentrations of 10^{-6} or 10^{-5} M, we applied NE for 5 min to test its effects. The BK response just before NE application was used as the control BK response, and the change from the control response in successive BK responses examined after NE application was taken as the change induced by NE. In the control experiments ($n = 8$), BK solution (10^{-5} M) was similarly applied 6 times at 10-min intervals without NE application.

Our results with BK were similar to previous results in normal Lewis rats [2]. A typical example of the effect of NE (10^{-5} M) on the BK response is shown in Fig. 1A. This CPR unit responded to three applications of BK (10^{-5} M) with a typical pattern of tachyphylaxis (BK1, -2, -3). NE (10^{-5} M) application just after the 3rd BK excited the unit to produce high-frequency discharges and clearly increased the subsequent BK response (BK4). This facilitating effect of NE was seen up to 10 min after the NE treatment (BK5). On average ($n = 7$), NE (10^{-5} M) application significantly augmented the subsequent CPR responses to BK (Fig. 2 ; $P = 0.02$, Wilcoxon test). An increased BK response was still observed 10 min later although with a slight tendency to decline (37.0 ± 16.5 impulses). This response was also significantly ($P = 0.02$, Wilcoxon test) larger than the control BK response. In 5 units observation was continued for another 10 min, and this declining tendency was confirmed (5.8 ± 5.3 impulses, sample shown in BK6, Fig. 1A). Lower concentration (10^{-6} M) of NE ($n = 5$) failed to facilitate the subsequent BK responses (Fig. 2), although the BK response of one CPR unit was increased (from 2 to 9 impulses). In the control ex-

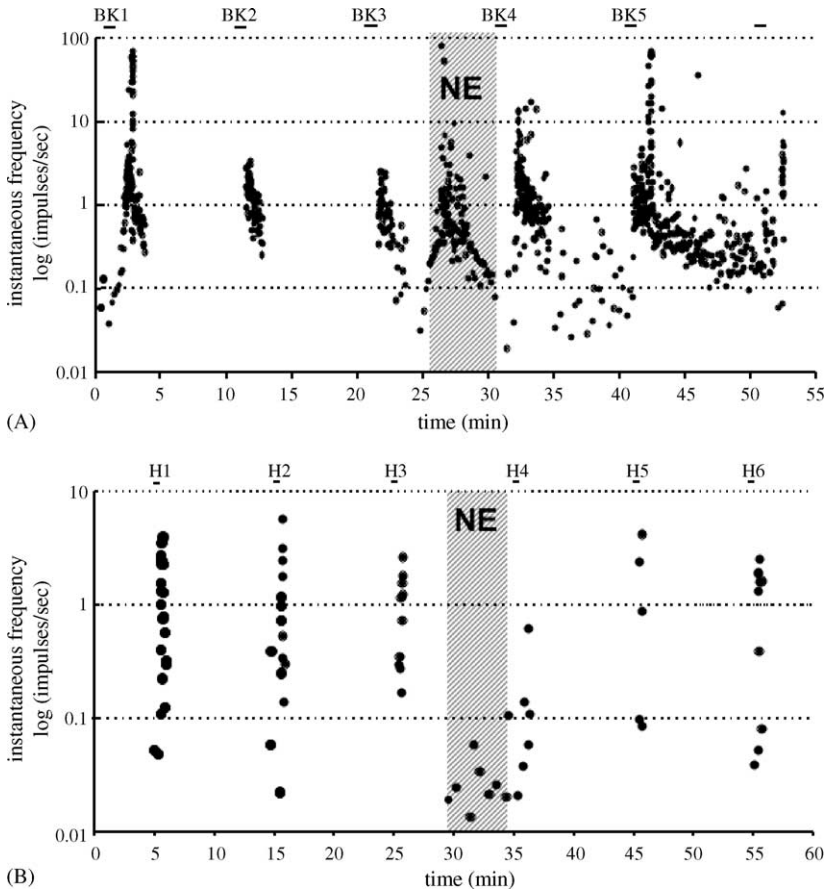

Fig. 1. Norepinephrine (NE) application induced excitation and facilitation of the bradykinin (BK) response (A) but inhibition of the heat response (B) in C-fiber polymodal receptors. Ordinate: instantaneous frequency of unit discharges (impulses/s) in log scale; abscissa: time (s). Lines indicate the time of 10^{-5} M BK (A) and heat (B) stimulation. NE (10^{-5} M) was superfused for 5 min (shaded area) before the 4th BK (BK4) or 4th heat stimulation (H4). Conduction velocity: 0.51 m/s (A); 0.45 m/s (B).

periment without NE application ($n = 8$), CPR responses to the 3rd and the 4th BK applications were similar in magnitude (without NE, Fig. 2).

NE (10^{-5} M) clearly augmented the BK response, similar to our previous observations [2]. We next addressed the question of whether NE would also influence the heat response of CPRs. In a similar preparation repeated heat stimulation was shown to activate polymodal nociceptive fibers to produce discharges of variable magnitude [8]. However, using the heating protocol with cut-off temperature described

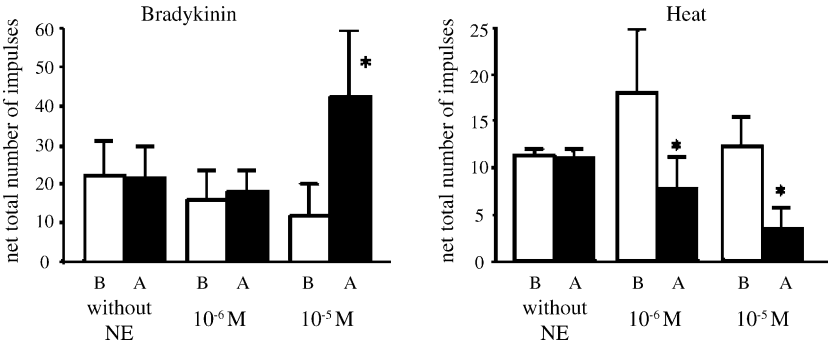

Fig. 2. Effects of norepinephrine (NE) application on the bradykinin (BK) and heat responses of C-fiber polymodal receptors. Average total numbers of discharges evoked by BK and heat stimulation before (B) and after (A) NE (10^{-6} or 10^{-5} M) application are shown as mean ± S.E. Without NE: data obtained from control experiments in which both responses were tested without NE application ($n = 8$). NE application at 10^{-5} M ($n = 7$) but not 10^{-6} M ($n = 5$) significantly increased the BK response ($^{*}P < 0.05$ vs. B, Wilcoxon test), while application at 10^{-6} M ($n = 12$) and 10^{-5} M ($n = 7$) significantly decreased the heat response ($^{*}P < 0.05$ vs. B, Wilcoxon test).

above CPRs were not sensitized or desensitized by repeated heat stimuli (unpublished observation). NE was applied after 3 applications of heat. The last heat response before the NE application was used as the control heat response, and the change from the control response in the heat response in successive examinations after the NE application was taken as the change induced by NE. A typical example of the effect of NE (10^{-5} M) on the heat response is shown in Fig. 1B. In the CPR unit shown in this figure, NE (10^{-5} M) application just after the 3rd heat application (H3) excited the unit with low-frequency discharges.

Contrary to the BK response, however, the heat response clearly and unexpectedly decreased after NE (H4). This inhibitory effect of NE still existed 10 min after the end of NE application (H5), finally disappearing after another 10 min (H6). This clear suppression of heat response following NE perfusion was seen in 6 out of 7 units tested with 10^{-5} M.

Even at an NE concentration of 10^{-6} M, which had no effect on the BK response (Fig. 2), suppressed heat responses were observed in 10 of 12 CPRs examined. On average, the heat response just after NE application at either concentration was significantly smaller than the control heat response (Fig. 2, 10^{-6} M: $P = 0.03$; 10^{-5} M: $P = 0.01$, Wilcoxon test). These inhibitory effects still existed in the response 10 min later. This inhibition was significant when the concentration was 10^{-5} M (7.0 ± 3.6 impulses, $P = 0.04$, Wilcoxon test) but not 10^{-6} M (10.6 ± 3.8 impulses). The response threshold temperature tended to rise after NE application at either concentration, although the changes were not significant (10^{-6} M: from 39.8 ± 1.1 °C to 41.0 ± 0.9 °C, 10^{-5} M: from 41.4 ± 2.0 °C to 43.0 ± 1.8 °C). In the control experiment without NE application, CPR responses to the 3rd and 4th heat applications were similar in magnitude (without NE, Fig. 2).

In the above experiments, we next examined the effect of repetitive BK or heat stimuli on the response of C-fibers to NE. Ten of the above CPR units received two applications of NE to their receptive field, one at least 60 min before any BK or heat stimuli and the other after 3 applications of BK or heat stimulations. The proportion of units excited by NE and the net total number of discharges induced by NE were then compared. None of the present units responded to NE at the concentration of 10^{-6} M ($n = 2$) or 10^{-5} M ($n = 8$) before BK or heat stimuli (Table 1). After BK stimulation, on the other hand, 1 out of 5 units (20%) responded to 10^{-6} M NE and 3 out of 7 units (43%) responded to 10^{-5} M NE (sample shown in Fig. 1A). Several heat applications also sensitized CPRs to NE (sample shown in Fig. 1B); 4 out of 12 units (33%) responded to 10^{-6} M NE and 2 out of 7 units (29%) responded to 10^{-5} M NE (Table 1). Thus, heat and BK had similar effects on the NE response.

Our previous study [3] showed that there were two distinct qualitative patterns of NE-excitation of CPRs: enhancement of ongoing discharges and short bursts of discharges. Both response patterns were also observed in the present experiment, and occurred similarly in NE responses after heat and BK stimuli. The responses of two 10^{-5} M NE-responsive units after BK trials were classified as enhancement of the ongoing discharges, and the pattern of these responses was similar to that of the previous BK response (sample shown in Fig. 1A). The response of one 10^{-5} M NE-responsive unit after heat also showed this pattern. CPRs without any spontaneous activities responded to NE with poor impulse generation and occasionally produced a burst of discharges. This pattern was seen in four 10^{-6} M NE-responsive units and three 10^{-5} M NE-responsive units after BK or heat trials (sample shown in Fig. 1B) in response to NE.

The magnitude of the NE response did not necessarily predict the magnitude of the subsequent BK or heat responses. Following NE, the BK response was the same or increased. Even in units not excited by NE, 50% of the following BK responses were sensitized (4 out of 8 units). More unusual was that in some units following NE excitation, the heat response was *decreased*. This suppression of the heat response after NE-induced excitation was observed in 50% of units (3 out of 6).

The present study demonstrated that NE application suppressed the subsequent heat responses of CPRs in normal conditions. This result agrees with a finding from in vivo recording of cutaneous C-fiber nociceptors in auricular nerve of rabbit: Sympathetic nerve stimulation suppressed sensitization in the following heat response [19]. Such sympathetic inhibition of the heat responses of C-fiber nociceptors has been considered to be a result of vasoconstriction, thus the skin temperature decrease. However, the present study was done in vitro, with the temperature of the receptive field kept constant. Even so, NE induced suppression of the following heat response. This finding suggests that suppression of the heat response by NE does not result from the tissue temperature decrease. Such suppression by NE of the subsequent heat response might imply that sympathetic nerve activities induced in exciting conditions (such as sport or religious ceremonies) suppress pain in the periphery.

Table 1
Incidence of responsive C-fiber polymodal receptors to norepinephrine (NE)

	Before bradykinin or heat		After bradykinin		After heat	
	n tested	*n* excited[a]	*n* tested	*n* excited[a]	*n* tested	*n* excited[a]
NE 10^{-6} M	2	0	5	1 (12)	12	4 (10–87)
NE 10^{-5} M	8	0	7	3 (10–33)	7	2 (23, 90)

n, number of fibers. In parentheses, the number of impulses during 5-min NE-superfusion are represented.
[a] See text for criteria.

This suppression of the heat response contrasts clearly with the increased response seen with BK. The increased BK response in this study with Sprague–Dawley rats agrees with the results from a previous study with Lewis rats [2]. Since the findings with BK in studies with different strains of rat were similar, it may also be reasonable to consider the suppressed heat response following NE in the present study to be a general phenomena, and not specific to one strain.

We previously demonstrated that α_2-adrenoceptors in C-fiber nociceptors are implicated in the sensitizing action of NE in a rat model of acute or persistent inflammation [3,17,20]. In normal rats the most common adrenoceptors in the dorsal root ganglion are of the α_{2C} subtype [23] and activation of α_2-adrenoceptors by NE is assumed to decrease intracellular cyclic AMP level [24]. In addition, raising intracellular cAMP facilitated heat response of visceral nociceptive fibers [10]. In view of these results, one may say tentatively that in the present study NE decreased cAMP through activation of α_{2C}-adrenoceptor subtype, and then subsequent heat responses were suppressed.

In the present study, a proportion of CPRs became sensitive to NE after both BK and heat stimulation. We can propose two hypotheses for NE-induced excitation after BK application. Firstly, repetitive BK stimuli produced a condition similar to inflammation of the tissue, possibly through production of prostaglandins [9] that sensitize CPRs to BK, heat and mechanical stimulation [7,11,13]. If this is the case, then prostaglandins may potentiate the NE effect to activate CPRs. Several lines of evidence support this explanation: Sympathetic nerve stimulation and exogenous NE increase the activities of nociceptors following sensitization by a heat injury [16] or the injection of a mixture of inflammatory mediators [6] as well as under an adjuvant-induced inflamed condition [3,20]. Second, exogenous BK may interact with the NE-releasing mechanism in sympathetic postganglionic terminals. This hypothesis is consistent with recent reports that the stimulation of sensory C-fibers by BK increased NE release from sympathetic terminals [21,22]. Taken together, this evidence suggests that BK increases the local NE concentration in the tissue, which enhances the magnitude of its response.

As a mechanism of NE-induced excitation after heat stimuli, repeated heat stimuli may produce inflammation of the tissue similar to the effect of repetitive BK application [14]. If this were true, NE would activate CPRs as described above. However, this hypothesis is unlikely, because in the present experiment the receptive field was stimulated using a heating protocol with cut-off temperature, and thus it is unlikely that the tissue was inflamed by the repeated heat stimuli. Alternatively, repetitive heat stimuli may have induced a subliminal inflamed condition after which NE actualizes tissue inflammation.

In conclusion, the present study provides evidence that after repetitive application of BK or heat, NE excites a proportion of C-fiber sensory units putatively involved in cutaneous pain. Moreover, whereas NE enhances the BK response of these nociceptive fibers, it suppresses the heat response regardless of the presence of NE-induced excitation. These results suggest different mechanisms of NE-modification of BK and heat responses.

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