

B2 Receptor–Mediated Enhanced Bradykinin Sensitivity of Rat Cutaneous C-Fiber Nociceptors During Persistent Inflammation

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Banik, Ratan Kumar, Yasuko Kozaki, Jun Sato, Lajos Gera, and Kazue Mizumura. B2 receptor–mediated enhanced bradykinin sensitivity of rat cutaneous C-fiber nociceptors during persistent inflammation. *J Neurophysiol* 86: 2727–2735, 2001. Bradykinin (BK), which has potent algescic and sensitizing effect on nociceptors, is of current interest in understanding the mechanisms of chronic pain. BK response is mediated by B2 receptor in normal conditions; however, findings that B1 receptor blockade alleviated hyperalgesia in inflammation have been highlighting the role of B1 receptor in pathological conditions. It has not yet been clear whether nociceptor activities are modified by B1 receptor agonists or antagonists during inflammation. In addition, previous studies reported the change in BK sensitivity of nociceptors during short-lasting inflammation, and data in persistent inflammation are lacking. Therefore we investigated whether an experimentally induced persistent inflammatory state modulates the BK sensitivity of nociceptors and which receptor subtype plays a more important role in this condition. Complete Freund's adjuvant was injected into the rat-tail and after 2–3 wk, persistent inflammation developed, which was prominent in the ankle joint. Using an in vitro skin-saphenous nerve preparation, single-fiber recordings were made from mechano-heat sensitive C-fiber nociceptors innervating rat hairy hindpaw skin, and their responses were compared with those obtained from C-fibers tested similarly in normal animals. BK at 10^{-8} M excited none of the 10 C-fibers in normal animals while it excited 5 of 11 (45%) C-fibers of inflamed animals, and at 10^{-6} M BK excited all of the 11 inflamed C-fibers (or 94% of 36 tested C-fibers) but only 4 of 10 (or 45% of 58 tested C-fibers) in normal animals. Thus the concentration-response curves based on the incidence of BK induced excitation, and the total number of impulses evoked in response to BK were significantly shifted to the left. Moreover, an increased percentage of the inflamed C-fibers responded to 10^{-6} M BK with bursting or high-frequency discharges. Thirty-percent of inflamed C-fibers had spontaneous activity, and these fibers showed comparatively less tachyphylaxis to consecutive second and third 10^{-6} M BK stimulation. A B2 receptor antagonist (D-Arg-[Hyp³, Thi^{5,8}, D-phe⁷]-BK) completely eliminated BK responses in inflamed rats, while B1 receptor antagonists (B 9958 and Des-Arg⁹-[Leu⁸]-BK) had no effect. Selective B1 receptor agonist (Des-Arg¹⁰-Kallidin) excited 46% ($n = 13$) of inflamed C-fibers at 10^{-5} M concentration, which is 1,000 times higher than that of BK needed to excite the same percentage of inflamed C-fibers. We conclude that in chronically inflamed tissue, sensitivity of C-fiber nociceptors to BK, which is B2 receptor mediated, is strongly increased and that B1 receptor may not

be important to a persistent inflammatory state, at least at the primary afferent level.

INTRODUCTION

Persistent inflammatory conditions commonly manifest with the increased pain due to noxious (hyperalgesia) and innocuous (allodynia) stimulation. Peripheral mechanism of these sensory phenomena is explained as the sensitization of primary afferent neurons supplying the tissue. Inflammatory chemical mediators such as bradykinin (BK), prostaglandin (PG) s, proton and others are responsible for activation and sensitization of primary afferent neurons. BK has attracted particular interest owing to its capacity to reduce behavioral nociceptive threshold (Taiwo and Levine 1988), to cause direct excitation of the nociceptors (Kanaka et al. 1985; Kumazawa and Mizumura 1980; Lang et al. 1990; Manning et al. 1991), and to sensitize nociceptors to mechanical (Neugebauer et al. 1989) and heat (Koltzenburg et al. 1992; Kumazawa et al. 1991; Lang et al. 1990) stimulation. BK and Kallidin (Lys-BK) or their precursor [high molecular weight (HMW) and low molecular weight (LMW)] kininogen levels are increased in both clinical and experimentally induced inflammation (Barlas et al. 1985, 1986; Hargreaves et al. 1988; Tsurufuji and Kumakura 1989). Increased sensitivity to BK was observed in nociceptors (Kirchhoff et al. 1990; Szolcsanyi 1987) in animal models of acute inflammation. There are two known receptors for kinins, B1 (described below) and B2. B2 receptor mediates the BK responses in normal conditions, and animals deficient of B2 receptors show hypoalgesia and reduced inflammatory responses (Boyce et al. 1996; Seabrook et al. 1997). Overall, previous work has established a good correlation between the inflammatory sensitization of nociceptors and BK. However, there are certain unresolved issues; one of which is the profound tachyphylaxis to BK on repeated application (Kanaka et al. 1985; Kumazawa and Mizumura 1980; Lang et al. 1990; Liang et al. 2001). This means that during prolonged exposure to BK, the excitatory effect will soon disappear. Therefore it might be interesting to know whether BK sensitivity is increased during persistent inflammatory conditions where noci-

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ceptors are continuously exposed to BK and whether there is a change in tachyphylaxis of BK response.

In addition, we also set out to clarify the exact role of different BK receptors under persistent inflammatory conditions. A number of previous studies, based on the observation of animal behaviors, suggested that under pathological conditions, de novo induction of B1 receptors take place, which plays a more significant role in nociception (Dray and Perkins 1993; Khasar et al. 1995; Perkins et al. 1993). These B1 receptors are activated by the selective endogenous ligand des-Arg⁹-BK (DABK) or des-Arg¹⁰-Kallidin (DAK), a naturally occurring metabolite of the parent BK or Kallidin. We have examined whether, under a persistent inflammatory state, DABK or DAK have any direct excitatory effect on nociceptors. Preliminary results have been published previously in abstract form (Banik et al. 1999).

METHODS

Animal model

Experiments were carried out on male Sprague-Dawley (SD) rats (SLC, Hamamatsu, Japan), 180–200 g at the beginning of the experiment. Polyarthrititis was induced by intradermal injection of 0.1 ml complete Freund's adjuvant (CFA), a suspension of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) in mineral oil (12 mg/ml), into the distal third of the tail (Colpaert et al. 1982; Pearson and Wood 1959). Two to 3 wk after inoculation of the CFA, rats developed decreased mobility, redness, and swelling of the hindpaw with other inflammatory signs. Rats that developed increased paw volume (measured by a mercury plethysmograph) were selected for single nerve fiber recording ($n = 33$). The naïve rats were used as controls ($n = 46$). Animals were kept under conventional animal facilities in a temperature-controlled environment with 12 h light/dark cycle. Particular care was taken with the regard of housing conditions. To minimize the discomfort of animals, rats that developed signs of inflammation were isolated into separate cages. A number of ethical considerations (Zimmerman 1983) for investigation of the experimental pain model were followed. First, the number of the inflamed animals was kept to a minimum. Second, outbred SD rats were chosen as they are affected less severely compared with inbred strain rats. Despite developing inflammation, the general condition of these animals (e.g., body weight gain) was not affected (Banik et al. 2001; Rosenthal 1970). All experimental procedures were approved by the Animal Care Committee, Research Institute of Environmental Medicine, Nagoya University.

Skin-nerve in vitro preparation

The details of the rat skin-nerve in vitro preparation have been described elsewhere (Reeh 1986). Rats were anesthetized with pentobarbital sodium (50 mg/kg). The saphenous nerve and its innervated territory on the hairy hindpaw skin was subcutaneously dissected until the nerve and skin could be removed. After dissection, rats were sacrificed with an intracardial injection of the high dose of pentobarbital sodium. The skin was placed "epidermal side down" in the in vitro perfusion chamber, and it was superfused with a modified Krebs-Hensleit solution (in mM: 110.9 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂SO₄, 24.4 NaHCO₃, and 20 glucose), which was saturated with a gas mixture of 95% O₂-5% CO₂ and the temperature of which was maintained at 34 ± 0.5°C. The saphenous nerve was drawn through a small hole into the recording chamber where aqueous solution was overlaid by a layer of paraffin oil. The nerve was placed on a mirror, and small filaments of nerve were repeatedly split with sharp forceps and thin needles until single-unit activity could be recorded.

Electrophysiological recording, thermal and mechanical stimulation

In this study we concentrated on the C-fiber nociceptors. Receptive fields of the units were identified by probing with a blunt glass rod in the corium side of skin. Conduction velocity of a fiber was determined by monopolar electrical stimulation (variable intensity, 0.2 Hz and 1 or 2 ms duration) into the receptive field. Then distance between receptive field and the recording electrode (conduction distance) was divided by the latency of the action potential. The mechanical threshold of units was tested with a set of calibrated von Frey hairs made from nylon filaments with uniform tips (0.5 mm diam). Heat responsiveness was examined by applying warm Krebs solution (50–55°C) at the end of experiment. The fiber that showed slowly adapting response to mechanical stimulation, responded to heat or BK and had conduction velocity ≤ 1.2 m/s was considered as C-fiber nociceptor in this study.

Action potentials were amplified, filtered, and displayed on an oscilloscope and continuously recorded on videotape (for off-line analysis) then processed on a personal computer using the analog-digital converter and SPIKE software package (a gift from Dr. Clemens Forster, University of Erlangen-Nuernberg, Germany).

Chemical stimulation and drug solutions

A metal ring (5.5 mm ID; height, 6 mm; volume, 0.4 ml) was used to isolate the receptive field (ring chamber). The chemical solution was superfused into the ring chamber at a speed of 2.6 ml/min. A thermocouple was placed within the ring chamber to monitor the temperature. The ring was emptied just prior to the arrival of the chemical solution into the ring chamber. Stock solutions (10⁻³ M) of BK and other drugs used were kept frozen (-80°C) and were diluted with the Krebs-Hensleit solution on the day of the experiment.

The following drugs were used for stimulation: bradykinin (BK), Des-Arg¹⁰-Kallidin (DAK), Des-Arg⁹-Leu⁸-BK (DALBK), D-Arg-[Hyp³-Thi^{5,8},D-Phe⁷]-BK (NPC 349), Lys-Lys-[Hyp³, Cpg⁵, D-Tic⁷, and Cpg⁸]-des-Arg⁹-BK (B 9958). Except B 9958 (Regoli et al. 1998), other drugs were purchased from the Peptide Institute, Minoh-Shi, Osaka, Japan.

Protocol of the experiments and criteria of responsiveness

BK or DAK was applied at 10-min intervals to the receptive field of a C-fiber by 10-fold increasing concentrations starting from 10⁻⁹ M to 10⁻⁶ M and in some cases to 10⁻⁵ M (protocol A). A concentration of more than 10⁻⁵ M was not used and if a C-fiber did not respond to 10⁻⁵ M, it was regarded as unresponsive to BK. DAK was always applied before BK. A C-fiber was considered to be responding to BK or DAK if at least 6 impulses were generated in response to a 1-min application. In other experiments (protocol B), 10⁻⁶ M BK was applied first to the receptive field of a C-fiber and if it showed sensitivity, then three to six consecutive applications were carried out. In a separate experimental series, B1 or B2 receptor antagonists were applied before the first application of 10⁻⁷ M BK and 15 min after the wash out of the antagonist, 10⁻⁷ M BK was given again. The effect of another antagonist was tested in the same unit if it showed sensitivity to 10⁻⁷ M BK, while protocol B was tried if it was unresponsive to 10⁻⁷ M BK. As the antagonist effect was always "all or none," "block" refers to a complete elimination of responses.

Data analysis

The magnitude of the BK responses of a C-fiber nociceptor was determined by counting the total impulses (action potentials) evoked during the 5 min after onset of BK superfusion. In all cases from the control rats and about 75% C-fiber from adjuvant rats, responses started and ended within this time window. For counting the total

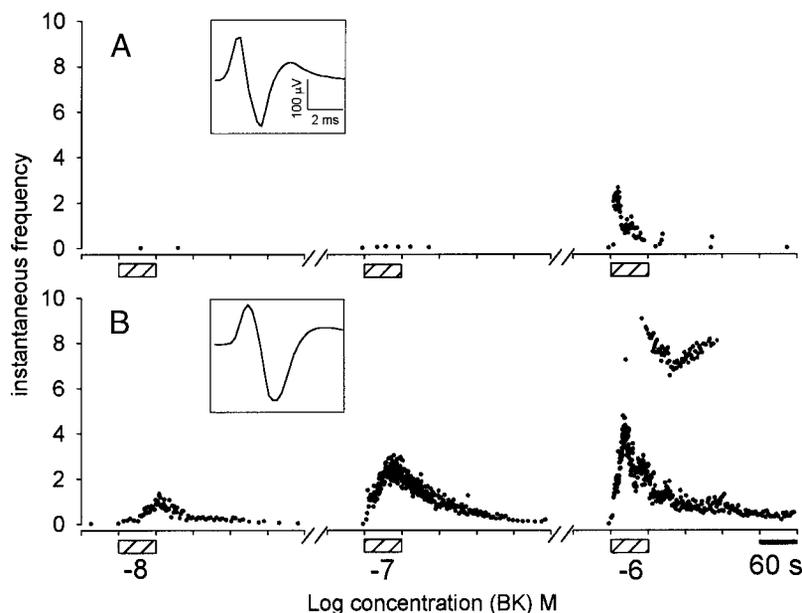


FIG. 1. Lowering of the bradykinin (BK) threshold concentration to excite C-fiber nociceptors during persistent inflammation. Specimen records from 2 single C-fiber nociceptors innervating control (A) and inflamed (B) rat hairy skin during consecutive trials of 10-fold increasing concentration of BK. Ordinate: instantaneous frequency of discharges (1/interval between spikes); BK was applied for 1 min (marked with obliquely hatched column) at 10-min intervals starting from 10^{-9} M to 10^{-6} M or 10^{-5} M. N.B.: 10^{-9} M and 10^{-5} M not shown. Insets display the action potential forms of these nociceptors.

impulses induced, spontaneous discharges during the 60-s control period were multiplied by 5 and then subtracted from the 5-min count after BK or DAK application. Data are presented as means \pm SE, unless otherwise stated. A χ^2 test or Fisher's exact probability test were used to compare the percentage of BK-responsive C-fiber, spontaneously discharging C-fiber and the pattern of BK excitation between inflamed and untreated control rats. The magnitudes of BK responses were compared using a nonparametric Mann-Whitney *U*-test. The normalized data of the effect of repeated 10^{-6} M BK applications (tachyphylaxis) were compared using a Student's *t*-test. For all tests, $P < 0.05$ was considered as significant.

RESULTS

General properties of C-fibers from inflamed and control animals

Ninety-four C-fiber nociceptors innervating the hairy skin of rat hindpaw were studied: 36 from the inflamed and remainder from the untreated control rats. The conduction velocities of the control C-fibers ranged from 0.1 to 1.0 m/s (0.6 ± 0.03 m/s, mean \pm SE), and those of the inflamed C-fibers were between 0.1 and 1.2 m/s (0.7 ± 0.04 m/s). Thirty percent of inflamed C-fibers (11/36) showed spontaneous activities without any intentional stimuli, which is significantly higher than 8% (5/58) of controls ($P < 0.006$, χ^2 test). There was also a significant increase in the discharge rates of spontaneous activity, which were between 0.05 and 0.7 imp/s (0.23 ± 0.06 imp/s) and 0.05 and 0.1 imp/s (0.07 ± 0.01 imp/s) when comparing the inflamed and control C-fibers, respectively ($P < 0.02$, Student's *t*-test). The mechanical threshold values of the C-fibers measured by von Frey hairs were a little lower in the inflamed animals (14.4 g/mm²; median), however, not significantly different from those of controls (20.25 g/mm²; $P > 0.08$, Mann-Whitney *U*-test). In inflamed C-fibers, no significant difference of the von Frey thresholds was observed between fibers with or without spontaneous activities ($P > 0.5$, Mann-Whitney *U*-test). In this study all tested C-fibers responded to the heat stimulation (50–55°C), and they had a single spot like receptive field.

Threshold concentrations of BK sensitivity

The threshold concentration of BK to excite the C-fibers was determined by application of BK at increasing concentration starting from 10^{-9} M and rising to 10^{-5} M (protocol A, Fig. 1; see METHODS). Of 10 control C-fibers, none responded to either 10^{-9} M or 10^{-8} M, and 2, 4, and 7 units responded to the 10^{-7} M, 10^{-6} M, and 10^{-5} M BK, respectively. As shown in Fig. 2A, a significantly increased proportion of C-fibers from the inflamed rats was sensitive to BK compared with controls: 2 at 10^{-9} M ($P > 0.15$, Fisher's exact probability test), 5 at 10^{-8} M ($P < 0.04$), 8 at 10^{-7} M ($P < 0.04$), and at 10^{-6} M BK all of the 11 fibers responded ($P < 0.004$). Four inflamed C-fibers had spontaneous activity; however, spontaneous activity did not influence the C-fiber sensitivity to the low concentration of BK. Results obtained from a different protocol confirmed this large difference in BK sensitivity. Under protocol B, 10^{-6} M BK was applied at first to the 25 inflamed C-fibers, and 23 units (92%) were detected to be responsive. The remaining two units responded to the successive application of 10^{-5} M BK. While

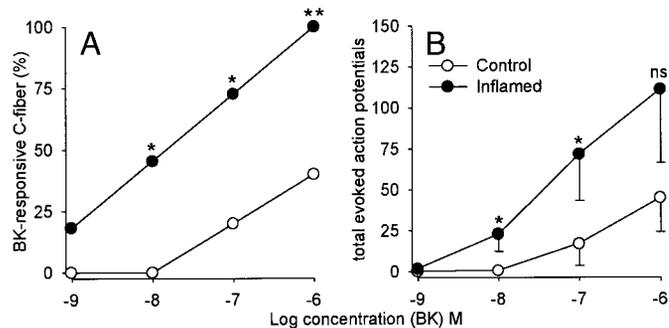


FIG. 2. Concentration-response curves of C-fiber nociceptors for BK. Significant differences were evident in (A) the incidence of BK sensitivity (* $P < 0.05$, ** $P < 0.005$, Fisher's exact probability test; $n = 10$, control and 11, inflamed) and (B) the mean of the total evoked action potentials by BK-responsive units (* $P < 0.05$, Mann-Whitney *U*-test; $n = 7$, control and 11, inflamed), between C-fibers from inflamed and control rats. A C-fiber was considered to be responsive to BK if at least 6 impulses were generated during 1-min superfusion. Total action potentials were determined by counting impulses evoked during 5 min after onset of BK-superfusion. ns, not significant.

using the same protocol, only 22 of 48 (about 46%) control units responded to 10^{-6} M BK; 10 unresponsive units were tested further with the 10^{-5} M, and 6 responded. The experimental variables like days after inoculation of CFA or the condition of the receptive field (sometimes, there were increased connective tissue in the receptive field of an inflamed preparation) had no impact on BK sensitivity.

BK response: magnitudes and patterns

In *protocol A*, the units monotonically increased their firing rate as the concentration of BK was increased (Fig. 1). Figure 2B shows a concentration-response relationship for the BK responses in both control and inflamed units treated under *protocol A*. Significantly increased BK-evoked discharges were detected in inflamed units at 10^{-8} M and 10^{-7} M ($P < 0.05$, Mann-Whitney *U*-test). As will be seen later, BK response undergoes tachyphylaxis when applied at 10-min intervals; therefore these might be the suppressed ones. When the average response magnitude to 10^{-6} M BK under *protocol A* was compared with that under *protocol B* (1st application), the former was smaller than the latter. However, the difference was not significant in both control (77.7 vs. 113.4 impulses; $P > 0.4$, Student's *t*-test) and inflamed cases (94 vs. 124.7 impulses; $P > 0.4$, Student's *t*-test).

In inflamed units, when the concentration increased from the 10^{-6} M to 10^{-5} M, units with initially large responses showed a smaller increase (for example, 25% increase from 455 imp/stimulus), while the units with small initial responses had a greater increase (for example, 500% increase from 21 imp/stimulus).

Control C-fibers generally responded with "slowly responding and low-frequency" discharges (Fig. 3A) while "rapidly responding and high-frequency" discharges (Fig. 3B) or "burst of discharges" (Fig. 3C) were typically observed in the inflamed C-fibers. The slowly responding and low-frequency

type of discharges started with a long latency ranging from 15 to 90 s and had low discharge rates (maximal frequency ≤ 3 spikes/s). Twenty controls (77%) and 8 inflamed (22%) C-fibers were classified in this category. The rapidly responding high-frequency type was labeled by the vigorous responses (maximal frequency 4–25 spikes/s) after a shorter latency (1–14 s), and it was predominant in the inflamed C-fibers when compared with controls (14/36, 41% vs. 4/56, 14%; $P < 0.03$, Fisher's exact probability test). The most distinct pattern of responses was the burst of discharges at a high-frequency, and it was observed in only two control C-fibers (7%), which responded with "spike doublets," but in 12 inflamed C-fibers (35%), which responded at 50.7 ± 10.0 spikes/s maximal frequency, 15.7 ± 11.6 s latency of the first burst and with the doublet (1 unit), triplet (2 units), or set of 4–15 impulses (9 units). One example of doublet is shown in Fig. 1B and of set of several spikes in Fig. 3C. The difference in the incidence of bursting discharges between two groups was also significant ($P < 0.03$, Fisher's exact probability test).

Tachyphylaxis of BK response

During repeated 10^{-6} M BK applications (10-min interval), control C-fibers (1st application; *protocol B*, $n = 14$) were excited by the second successive application with considerably decreasing discharges (Fig. 4A), and then adapted within three to six applications to a stable state (data not shown). The spontaneously discharging inflamed C-fibers ($n = 7$) showed a different behavior as in a sample recording in Fig. 4B, namely, less decay of the successive 10^{-6} M responses when compared with the controls (Fig. 4C). Interestingly, 12 inflamed C-fibers, those that had no spontaneous activity, responded in the same manner as control units (Fig. 4C). Even up to the sixth application, in no cases did the discharges become zero. With the exception of one unit from inflamed skin, the first response to a series of BK (10^{-6} M) stimuli was always the strongest.

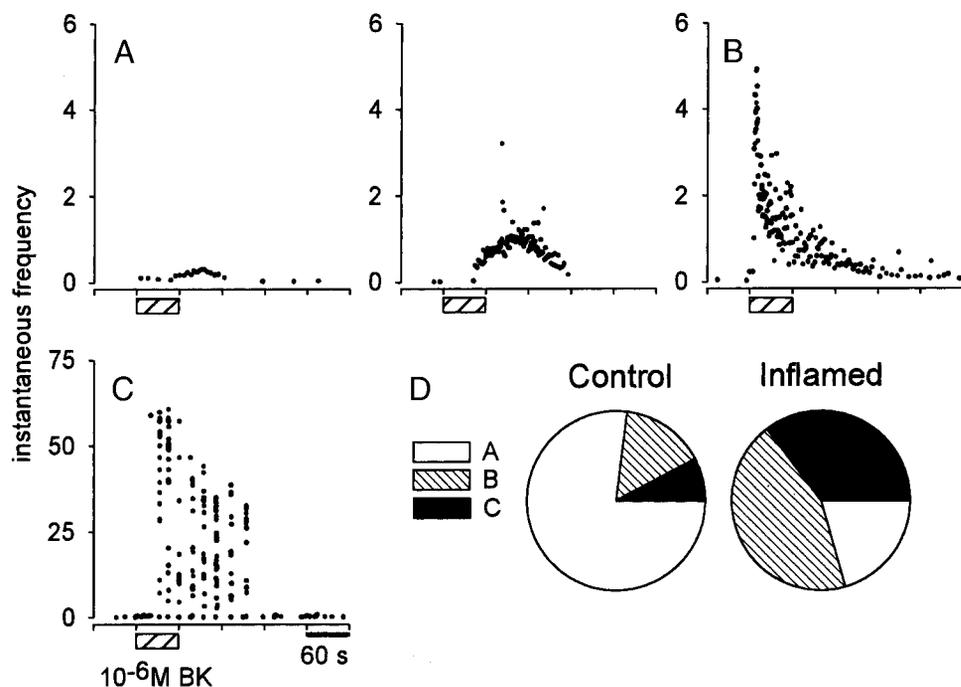


FIG. 3. Response patterns evoked by 10^{-6} M BK. Sample recordings were taken from 4 single C-fibers innervating the control (A) and inflamed (B and C) rat skin, which responded to 10^{-6} M BK with different patterns of discharges: slowly responding and low-frequency (A), rapidly responding and high-frequency (B) and burst of discharges (C). See text for criteria of these patterns. Ordinate: instantaneous frequency of discharges. Note that range in ordinate of pattern C is very large compared with other types. D shows the incidence of these 3 response patterns. Pattern B and C were significantly predominant ($P < 0.05$, Fisher's exact probability test) in the inflamed C-fibers ($n = 34$) compared with controls ($n = 26$).

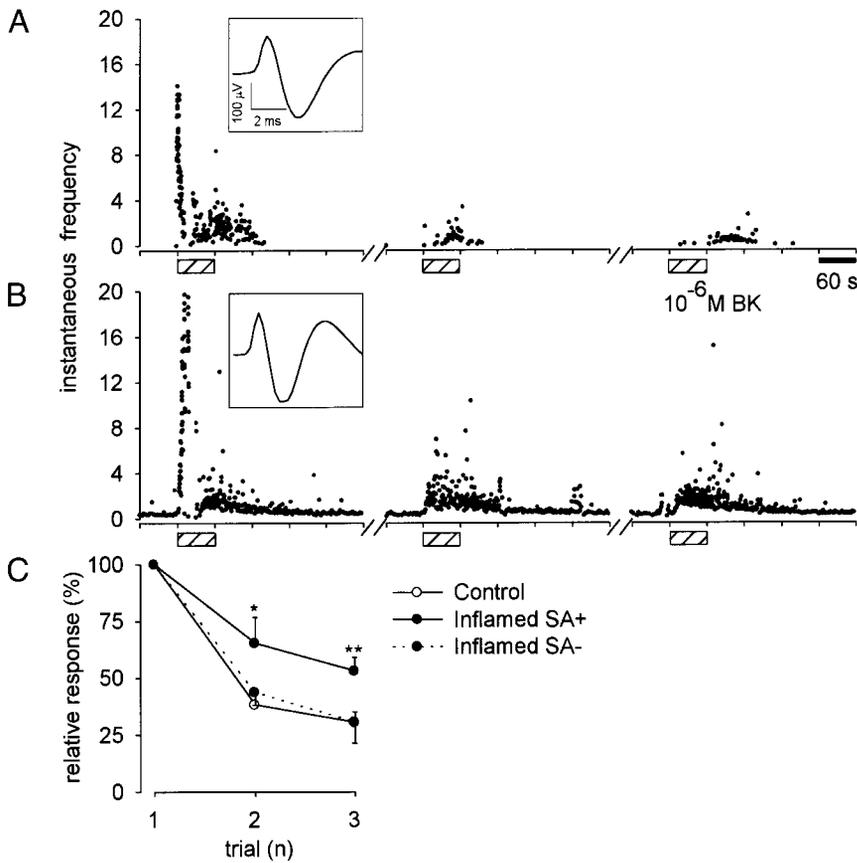


FIG. 4. Tachyphylaxis of BK response in control and inflamed rats. Continuous recording were taken from 2 single C-fibers supplying the control (A) and inflamed (B) rat skin during repeated BK (10^{-6} M) applications at 10-min intervals. BK application period is marked with obliquely hatched column. Insets display action potential forms of these nociceptors. Ordinate: instantaneous frequency of discharges. C: magnitude of C-fiber responses during consecutive trials of 10^{-6} M BK. Data normalized to the first response. C-fibers from inflamed rats were classified as with (SA+) or without (SA-) spontaneous activity. * $P < 0.05$, ** $P < 0.005$, Student's *t*-test; ns, not significant. $n = 14, 7,$ and 12 for control, inflamed SA+, and inflamed SA-, respectively, tested up to 3 consecutive applications.

Increasing the BK concentration (Fig. 2B) and prolonging the interval (1 h) of successive 10^{-6} M BK stimulation could reverse the tachyphylaxis tendency in both control and inflamed C-fibers. Extent of tachyphylaxis was not different for units with large initial responses (>75 imp/5 min) and those with comparatively small responses (average percent fall of responses to 2nd application, 36 vs. 45% for control and 51 vs.

51% for inflamed rats in units with large and small initial responses, respectively).

Role of BK receptor subtypes in inflamed C-fibers

The effects of B1 and B2 receptor antagonists on BK responses indicated that BK sensitivity of the inflamed C-fibers is mediated by B2 receptors. This observation was made from

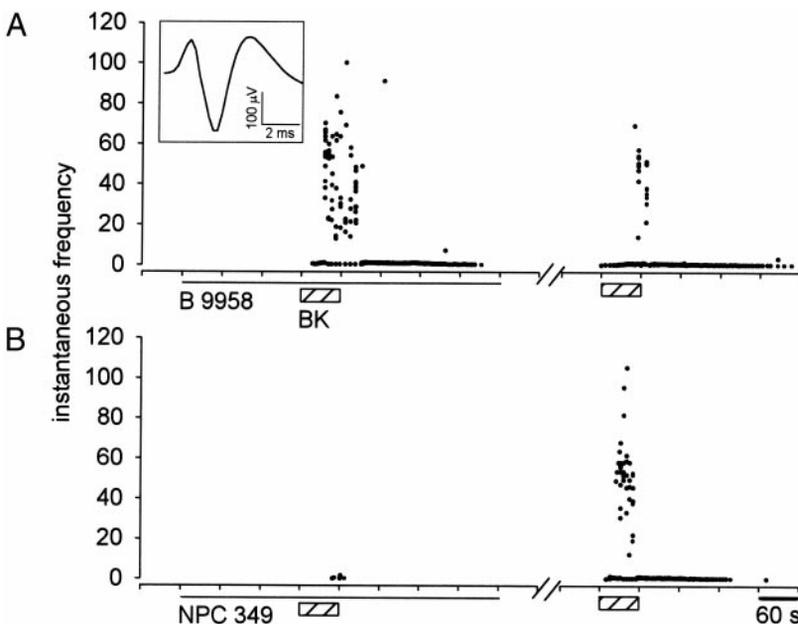


FIG. 5. B2 receptor mediation of the BK response in a C-fiber from an inflamed rat. A: in the presence of 10^{-5} M B 9958, a long-acting B1 receptor antagonist 10^{-7} M BK evoked a clear response, and the successive response of 10^{-7} M BK was reduced in comparison, suggesting that the B1 receptor antagonist had no effect on BK response. B: in the presence of 10^{-5} M NPC 349, a short-acting B2 receptor antagonist, 10^{-7} M BK failed to excite the unit, and, after wash out of the NPC 349, BK evoked clear excitation suggesting that the B2 receptor antagonist completely blocked the BK response. Application periods for antagonists and BK are marked by solid line and obliquely hatched column, respectively. Relative times of 4 10^{-7} M BK applications are 0, 15, 45, and 60 min. Inset displays action potential form of this nociceptor. Ordinate: instantaneous frequency of discharges.

11 BK (10^{-7} M) sensitive (proved by 10^{-7} M BK responses 15 min after wash out of the antagonist) fibers from inflamed rats. The first application of 10^{-7} M BK was challenged by antagonists and the consecutive 10^{-7} M BK (15-min interval) responses were used to determine the BK sensitivity. In the presence of NPC 349 (10^{-5} M), a competitive and short-acting B2 receptor antagonist, no inflamed units ($n = 7$) responded to BK (10^{-7} M; Fig. 5B). The average total responses evoked in the presence and absence of NPC 349 was 1.2 ± 0.7 and 73.7 ± 28.0 impulses, respectively. Five fibers were challenged with the long-acting B1 receptor antagonist, B 9958 (10^{-5} M) and the clear BK responses were observed, although concentration of the antagonist was 100 times higher than BK (Fig. 5A). All these fibers were excited by the follow-up application of BK (10^{-7} M). The average total responses evoked in the presence and absence of B 9958 was 92.2 ± 35.6 and 87.75 ± 30.9 impulses. The extensively used B1 receptor antagonist DALBK (10^{-5} M) was also tried in three units, and in the presence of DALBK one unit was excited by BK (10^{-7} M). DALBK itself excited the remaining two units, which concealed their responses to BK. Unlike DALBK, NPC 349 and B 9958 had no effect on the development or rate of spontaneous activity when used up to 10^{-5} M concentration.

Selective B1 receptor agonist induced excitation in inflamed C-fibers

None of the six control C-fibers responded to the selective B1 receptor agonist, DAK at 10^{-5} M concentration. In contrast, this agonist weakly excited the inflamed C-fibers in a concentration-dependent manner, although starting from a higher concentration (10^{-6} M) compared with BK. One representative sample is shown in Fig. 6A. At the highest concentration of DAK (10^{-5} M) used, 6 of 13 C-fibers responded (46%, Fig. 6B). The average of the total impulses evoked by DAK in individual C-fibers was also much lower than that of BK (Fig. 6C). DAK-induced weak firing was challenged by the B1 receptor antagonists in several experiments. Unfortunately, due to the excitation caused by B1 receptor antagonists B 9958 or DALBK themselves at a concentration more than 10^{-5} M, these results were impossible to interpret. In two DAK (10^{-5} M) responsive inflamed C-fibers, DALBK at the same concentration as DAK apparently blocked the DAK-induced excitation. Fifteen minutes after wash out of DALBK, one unit was excited by DAK, and the other produced a few impulses.

DISCUSSION

Enhanced BK sensitivity

In the present experiments we have described the leftward shift of BK concentration-response curve of C-fiber nociceptors innervating the rat hindpaw skin during persistent inflammation. Authors of previous investigations in short duration of inflammation (3 h to 1 day) have concluded, based on the effect of single concentration of BK, that nociceptors in inflamed tissue developed responsiveness, or responded with increased magnitudes to BK (Kirchhoff et al. 1990; Koltzenburg et al. 1999; Szolcsanyi 1987). A noteworthy finding of the present study is the lowering of the BK-threshold concentrations during persistent inflammation. Two of 11 inflamed C-fibers re-

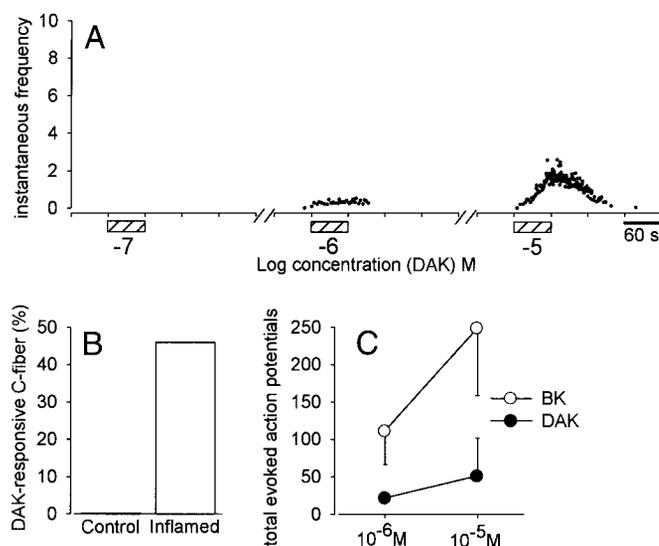


FIG. 6. Effects of B1 receptor agonist, Des-Arg¹⁰-Kallidin (DAK) on C-fibers from control and inflamed rats. A: instantaneous frequency plots during consecutive trials of 10-fold increasing concentration of DAK on a C-fiber from an inflamed rat (the same unit that showed in Fig. 1B). DAK application periods are marked with obliquely hatched column. Ordinate: instantaneous frequency of discharges. B: incidence of the 10^{-5} M DAK-responsive C-fibers in control ($n = 6$) and inflamed ($n = 13$) rats. C: DAK-evoked total action potentials are compared with those of BK in inflamed rats. Criteria for responsiveness and calculation of the total evoked action potentials are described in Fig. 2.

sponded to 1 nM BK, which is the lowest concentration of BK for activation of nociceptors in any preparations (see review, Mizumura and Kumazawa 1996).

It is possible that BK response of the inflamed C-fibers can be sensitized by other mediators such as PGs. PG E2 and I2 are the powerful sensitizing substances for BK responses (Kumazawa et al. 1996; Lang et al. 1990; Mizumura et al. 1991), and their levels are known to increase in the inflamed tissue. A recent report by Segond von Banchet et al. (2000) and the unpublished observation of our laboratory raised the possibility of BK receptor up-regulation. Using a nanogold method, Segond von Banchet et al. (2000) showed an increased expression of B2 receptors in dorsal root ganglion (DRG) neurons of rats rendered arthritis of the knee joints for up to 6 wk. In agreement, the unpublished data of our laboratory has also shown an increased expression of B2 receptor mRNA (observed up to 3 wk) in the DRGs of the presently used animal model (Kozaki et al. 2000).

Because in this chronically inflamed rat skin BK receptors have been continuously exposed to increased concentration of BK (Hargreaves et al. 1988), the up-regulation of B2 receptor is somewhat contradictory to a fundamental property of the most G-protein-coupled receptors, namely, downregulation by agonist. One consideration may be nerve growth factor (NGF), which has been shown to increase the BK sensitivity of small DRG neurons during persistent inflammation (Kasai and Mizumura 1999) and to increase the expression of B2 receptors on cultured DRG neurons from adult mice via p75 receptor (Petersen et al. 1998). The levels of endogenous NGF are substantially increased in the inflamed tissue (Donnerer et al. 1992).

BK response patterns

A significantly increased percentage of the inflamed C-fibers responded to 10^{-6} M BK with bursting or rapidly responding and high-frequency discharges in inflamed skin (Fig. 3D). This observation indicates the alteration of the membrane properties of inflamed C-fibers. A recent observation provides support for this by showing that CFA treatment induced functional changes in the C-fiber DRG perikarya: shorter duration of the action potential and decreased action potential rise and fall time (Djoughri and Lawson 1999). Such changes might allow repetitive firing at higher than normal frequencies. It is possible that similar changes occur in C-fiber terminals since the properties of soma and fiber membrane show particular similarities (Harper 1991).

Tachyphylaxis to BK stimulation

BK responses undergo strong tachyphylaxis during repeated stimulation in most preparations including the one used in the present study. In our study, BK-response of the inflamed C-fibers that had no spontaneous activity showed a similar tachyphylaxis tendency as control C-fibers. BK response showed tachyphylaxis also in the spontaneously discharging inflamed C-fibers, however much less than normal (Fig. 4C). These findings are partly in agreement with the Kirchoff et al. (1990), who reported that all fibers innervating an acutely inflamed rat responded readily to the second and third 10^{-5} M BK stimulation compared with only 50% of their controls.

Spontaneous discharges of nociceptors are linked to the abnormal activation of the kinetically slow, tetrodotoxin-resistant (TTX-R) Na^+ channels. It has been reported that alterations in levels of the TTX-R Na^+ channels (Schild and Kunze 1997) or up-regulation of preexisting channels (Gould et al. 1998; Tanaka et al. 1998) occurs after inflammation. After axotomy, NGF is reported to play a key role in modulating the TTX-R Na^+ channel expression in DRG (Black et al. 1997), and neutralization of the endogenous NGF abolishes spontaneous activity of C-fibers innervating carrageenan-inflamed skin (Koltzenburg et al. 1999). These observations led to speculation that increased NGF level in the inflamed tissue can selectively inhibit BK desensitization in spontaneously discharging nociceptors. An alternative possibility is PGE₂, which is present in increased amount in inflamed tissue, is well-known to sensitize BK response, and is also involved in developing spontaneous activity (Heppelmann et al. 1986). It is possible that these mechanisms occur, but to different degrees in different preparations.

Role of BK receptor subtypes

Our results show that B2 receptor activation is the major mechanism by which BK activates the primary afferent neurons during persistent inflammatory conditions. In addition, we have provided the first electrophysiological evidence for an effect of a selective B1 receptor agonist (DAK) on nociceptors innervating an inflamed tissue. It should be noted, however, that the concentration of DAK required was almost 1,000 times higher than for BK. As we could not convincingly show whether the DAK-evoked small response was at all mediated by the B1 receptor, there is a possibility that the action of DAK

is mediated by B2 receptor, which is substantially increased in an inflammatory condition identical to the present study (Segond von Banchet et al. 2000). In agreement with our data, Kasai et al. (1998) has shown that DAK had no effect on membrane potentials of small DRG cells cultivated with NGF for 2 days. Likewise, Davis et al. (1996) did not observe any DAK effect in DRG neurons even after treatment with interleukin- 1β , PGE₂, and PGI₂. These observations including the present data do not support the suggested involvement of B1 receptor in nociception during persistent inflammation (Ahluwalia and Perretti 1999; Calixto et al. 2000; Dray and Perkins 1993). It is worth noting that this study could not rule out the involvement of B1 receptor in nociceptive pathway other than the primary afferent level since it used an *in vitro* preparation. There is a possibility that B1 receptor is involved in the spinal cord in light of the recent reports that B1 receptor is located in that place (Raidoo and Bhoola 1997) and that hypoalgesia of B1 receptor-deficient mice is due to the reduction of B1 receptor activity in the spinal cord (Pesquero et al. 2000).

Possible relevance to inflammatory hyperalgesia of BK hypersensitivity

In the present experiments, we did not see any decrease of the mechanical threshold after inflammation. Recent evidence has shown that inflammation changes the suprathreshold sensitivity of nociceptors to mechanical stimulation, although thresholds are not altered (Andrew and Greenspan 1999). Inflammatory mediators that contribute to mechanical sensitization of nociceptors have not been well studied. BK and PGE₂ sensitize to mechanical stimulation of about 70 and 50% of the single afferents from normal joints, respectively, and the majority of these joint units sensitized when a combination of BK and PGE₂ was given (Neugebauer et al. 1989). Recent data from our laboratory indicate that the response magnitudes of testicular nociceptors to mechanical stimulation are enhanced by BK (Mizumura and Koda 2000). These results provide support for the view that BK hypersensitivity of nociceptors is capable of contributing to mechanical sensitization after inflammation.

BK sensitizes the nociceptors to heat stimulation (Kumazawa et al. 1991; Lang et al. 1990; Liang et al. 2001), and sensitization to heat after strong application of heat (Mizumura et al. 1992) is believed to be caused, to a degree, by endogenous BK (King et al. 1976). Therefore the enhanced BK hypersensitivity may account for heat hyperalgesia in the inflamed tissue (Raja et al. 1999).

Finally, this study provides the first hints that an underlying cause of pain and hyperalgesia during chronic inflammatory diseases such as rheumatoid arthritis may be enhanced BK sensitivity. If up-regulation of the B2 receptors is responsible for such hypersensitivity as shown in a recent report, modulation of the B2 receptor up-regulation can be considered as a novel approach for chronic pain therapy.

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