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Increased Expression of mRNA for B1 and B2 Bradykinin Receptors in the Skin of Adjuvant Inoculated Rats

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Abstract: Bradykinin is the most established endogenous algesic substance. It has two receptors subtypes, B1 and B2, which are classified based on their pharmacological properties. It is generally accepted that the B2 receptor (B2R) is normally expressed in many tissues, whereas the B1 receptor (B1R) exists only in limited tissues and is synthesized *de novo* following tissue injury. We recently demonstrated that B2R-mediated bradykinin responses of peripheral nociceptors were increased in skin-saphenous nerve preparations *in vitro* in adjuvant inflamed rats, but involvement of B1R could not be confirmed. To examine the possibility that B1R is expressed in cells other than neurons and that we missed B1R-mediated responses while using in vitro preparations, we evaluated the mRNA expression of B1R in rat hind-paw skin and compared it with that of B2R after adjuvant inoculation. B2R mRNA was expressed in control and inflamed animals, and the level of its expression was increased 3 weeks after adjuvant inoculation. B1R mRNA, in contrast to the general belief, was expressed in control animals and increased 3 weeks after adjuvant inoculation, similar to B2R mRNA. Possible meanings of this finding are discussed.

Key words: bradykinin, B1 bradykinin receptor, B2 bradykinin receptor, mRNA expression, arthritic rat

In various morbid states including injury and inflammation, the pain sensation is augmented (hyperalgesia) and weaker stimuli which do not usually produce pain become painful (allodynia). Inflammatory mediators such as bradykinin, histamine, prostaglandin E_2, and serotonin are reported to be increased in inflamed tissues. Of these inflammatory mediators, bradykinin is the most established endogenous algesic substance and its stimulating effects on nociceptors have been documented in the skin,[1] muscle,[2] and viscera.[3] There are two subtypes of bradykinin receptors, B1 and B2, based on their pharmacological properties.[4] The B2 receptor (B2R) is normally expressed in many tissues, whereas the B1 receptor (B1R) is believed to exist only in limited tissues and to be synthesized *de novo* following tissue injury.[5] Since B1R antagonist suppresses pain behavior in UV- or adjuvant-induced inflammation, it has been supposed that B1R mediates hyperalgesia in inflammation.[6] We demonstrated that B2R-mediated bradykinin responses of peripheral nociceptors were increased in skin-saphenous nerve preparation *in vitro* in adjuvant inflamed rats.[7] However, we could not confirm that B1R was involved in nociceptor excitation in this preparation. Several investigators tried to find a nociceptor response to B1R agonist in cultured dorsal root ganglion (DRG) neurons, but they failed.[8,9] These results led to the hypothesis that B1Rs are expressed in cells other than neurons.

In the present study, we examined this hypothesis by evaluating the mRNA expression of B1R in inflamed skin where neuronal cell bodies do not exist, and compared it with that of B2R after adjuvant inoculation, using reverse transcription (RT) and polymerase chain reaction (PCR).

Materials and Methods

Animal treatment and skin sampling

Male Sprague-Dawley (SD) rats (SLC Inc.,Japan) were used (body weight: 180–200 g at the beginning of the experiment, n=42). The animals were housed three per cage and maintained under a 12-h light/dark cycle in a temperature-controlled animal house. Food and water were available ad libitum. The animals were randomly divided into two groups. One group was untreated and served as the control and the other group was subjected to adjuvant-induced inflammation. Adjuvant inflammation was induced by the method reported previously.[10,11] Briefly, the rats were anaesthetized with diethyl ether and injected with 0.1 ml complete Freund's adjuvant (CFA), a suspension of heat-killed *Mycobacterium butyricum* (Difco Inc MI) in paraffin oil (12 mg/ml), in the distal third of the tail. The region of hind-paw skin innervated by the saphenous nerve was subcutaneously removed under anesthesia with sodium pentobarbital (50 mg/kg) at the time points described

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below. Because CFA induces inflammation in only 40% of rats, and inflammation develops about 2 weeks after inoculation,[12] specimens for the control and 1 week after inoculation were taken from all the corresponding rats, and specimens for 2 and 3 weeks after inoculation were taken only from rats that showed signs of inflammation (n=6 per time point). Several DRGs were dissected from each animal; in addition, prostates were dissected as the positive control. These tissues were immediately frozen in liquid nitrogen and stored at –80°C until RNA extraction was done. To confirm the influence of contaminated blood, some skin samples from animals at 2 weeks after inoculation were perfused with aerated Krebs solution over 2 hours before freezing.

All experimental procedures were approved by the Animal Care Committee, Research Institute of Environmental Medicine, Nagoya University.

RNA extraction and RT

Total RNA was extracted from homogenated skin by the acid guanidinium thiocyanate-phenol-chloroform method.[13] The RNA was then treated with RNase-free DNase I (Promega, Madison, WI, USA) in order to digest the genomic DNA, followed by extraction with neutral phenol/chloroform. The amount of RNA was determined by optical density at 260 nm. Total RNA was reverse-transcribed using oligo dT(16) primer and 200 U of M-MLV reverse transcriptase (Promega).

PCR and semi-quantitative analysis

PCR amplification of mRNA for B1 (B1R) and B2 (B2R) receptors, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using the RT products as templates. The sequences of oligonucleotide primers for B1R, B2R and GAPDH are shown in Table 1. The PCR conditions for B1R, B2R and GAPDH mRNA were as follows: initial denaturation at 94°C for 3 min followed by appropriate cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. To confirm that the amplified fragments were derived from B1R and B2R mRNA, the stocks of PCR products were digested with restriction enzymes, PvU II (for B1R) and Hind III (for B2R).

Preliminary experiments showed a linear non-saturating increase in amplification products between 35 and 41 cycles for the B1R, between 32 and 38 cycles for the B2R, and between 18 and 24 cycles for the GAPDH in the skin sample. Unless otherwise stated, PCR was run for 38 cycles for B1R, 36 cycles for B2R and 24 cycles for GAPDH. Portions of PCR product mixtures were electrophoresed in an agarose gel and stained with ethidium bromide. The densities of the bands were quantified using the image analysis software Scion Image, version 4.0.2. The results for the BK receptors were normalized for the amount of GAPDH measured simultaneously in each sample.

Statistical analysis

Data are presented as mean ± SE. The data were analyzed using either Bonferroni's multiple *t*-test or Student's *t*-test. Differences were considered significant if $p<0.05$.

Results

One week after CFA injection, we observed signs of inflammation localized in the skin of the injection site (at the distal third of the tail). Two and 3 weeks after the injection, we observed inflammatory signs such as decreased mobility, redness and swelling of the hindlimb below the knee, foot deformity and other signs of generalized inflammation in 16 out of 30 animals injected with CFA. Specimens for 2–3 weeks after inoculation were taken only from animals with inflammatory signs.

RT-PCR of B1R and B2R mRNA was performed using 1 μg of total RNA samples from the rat hind-paw skin. It gave clear single bands corresponding to 481 and 553 bp, which were predicted from the sequence of B1R and B2R mRNA. The digestion of the PCR product for B1R by restriction enzyme PvU II resulted in two bands at sizes predicted from the

Table 1. Primer pairs used

Gene	Primer	Sequence	Position	Predicted size (bp)	GenBank
B1R	sense	5'-GTGGTCAGCGGGGTCATCAAGG-3'	512 – 533	481	U66107
	antisense	5'-GGAAAGCGAAGAAGTGGTAAGG-3'	971 – 992		
B2R	sense	5'-GCCTTCCGGATGGTTTCA-3'	2970 – 2987	553	M59967
	antisense	5'-TGGTGTTGGAGGTTGTTTGATA-3'	3501 – 3522		
GAPDH[a]	sense[b]	5'-GTGAAGGTCGGTGTCAACGGATTT-3'	34 – 57	555	M17701
	antisense[b]	5'-CACAGTCTTCTGAGTGGCAGTGAT-3'	565 – 588		

a): Glyceraldehyde 3-phosphate dehydrogenase; b) These primer sequences were described by Andoh et al.[14]

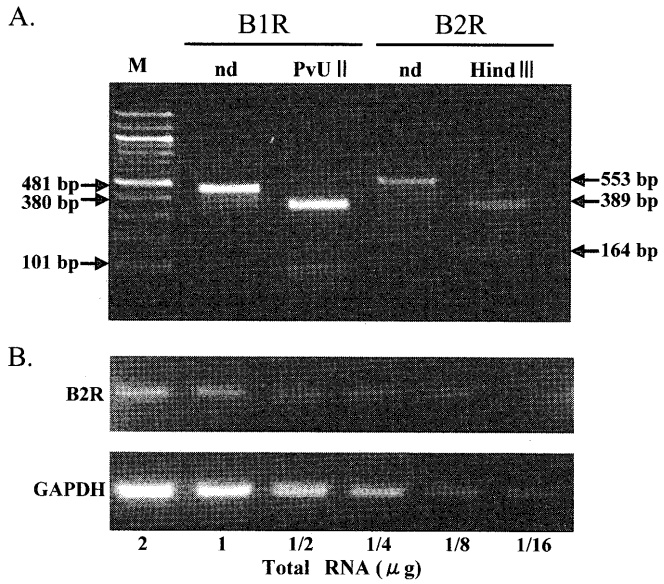

Fig. 1 Reverse transcription and polymerase chain reaction (RT-PCR) of B1 (B1R) and B2 bradykinin receptor (B2R) mRNA.
A. Digestion of PCR products by restriction enzymes.
PCR products of B1R and B2R mRNA from the rat skin were digested by restriction enzymes, PvU II for B1R and Hind III for B2R. M: 100 bp DNA ladder; nd: not digested. The PCR products before digestion were detected as clear single bands with the sizes predicted (481 bp for B1R and 553 bp for B2R) and the digestion gave the bands with predicted sizes (380 and 101 bp for B1R, and 389 and 164 bp for B2R).
B. RT-PCR of serially diluted total RNA.
RT-PCR of B2R and GAPDH mRNA was examined using serially diluted total RNA (1/16-2 μg) from the rat dorsal root ganglia. The PCR was run for 33 cycles for B2R and 21 cycles for GAPDH. In this dilution range, differences in densities of the bands between two adjacent dilutions could be discriminated.

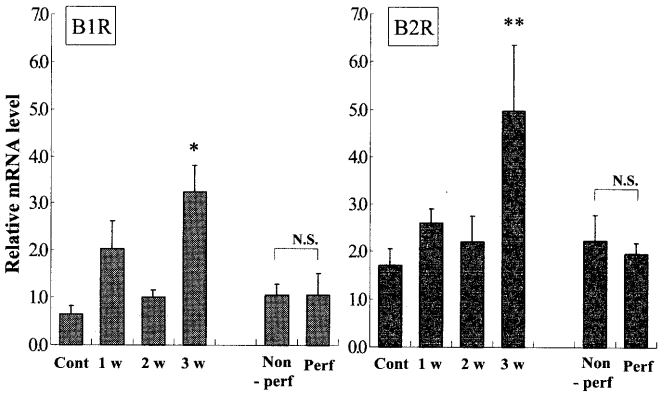

Fig. 2 Changes in mRNA expression of B1R (left graph) and B2R (right graph) in the rat hind-paw skin.
RT-PCR products of B1R and B2R mRNA from the hind-paw skin were examined in the control and the adjuvant-inflamed rats. Cont: control, n = 6; 1w, 2w and 3w: 1, 2, and 3 weeks after inoculation of complete Freund's adjuvant, n = 6 per group; Perf: skin samples were perfused with Krebs solution from rats 2 weeks after the inoculation, n = 6; Non-perf: without perfusion from rats 2 weeks after the inoculation, n = 6. Each column and bar represent the mean and SE. * $P < 0.05$, ** $P < 0.01$ by Bonferroni's multiple *t*-test compared with control, respectively. N.S. indicates no significant difference by paired *t*-test.

sequence of mRNA, at 380 and 101 bp. The digestion of PCR product for B2R by Hind III gave two bands corresponding to 389 and 164 bp, as predicted. These results suggest that the amplified fragments were derived from B1R and B2R mRNA.

To ascertain the possibility of semi-quantitative PCR analysis, total RNA samples from the DRG and prostate were doubly diluted and assayed for B2R and B1R mRNA. Differences in densities of the bands of B2R between two adjacent dilutions could be discriminated in the range of at least 1/16 μg to 2 mg of total RNA (Fig. 1B), and those of B1R were discriminable in the range of 1/4 μg to 2 μg of total RNA (data not shown). Therefore, 1 μg of total RNA was used for the RT-PCR in the following experiments.

The changes in expression of B1R and B2R mRNA in the skin samples of the control and inflamed rats are shown in Fig. 2. B2R mRNA was detected in the skin of both control and inflamed animals with large variations. Although the expression of B2R in inflamed animals appeared to be greater than in control animals at all times, the increase was statistically significant only at three weeks after inoculation ($P < 0.01$, by Bonferroni's multiple *t*-test), which was one week after the appearance of signs of inflammation such as redness of skin and swelling.

B1R mRNA was detected in the skin of inflamed animals, and unexpectedly, it was also detected in the skin of the control animals with large variations. The increase was significant only at three weeks after the inoculation ($P < 0.05$, by Bonferroni's multiple *t*-test).

Excised skin was perfused with Krebs solution to wash out the circulating immunocytes. This procedure did not change the mRNA levels at all (paired *t*-test).

Discussion

In this study, systemic inflammation was observed at 2 and 3 weeks after CFA inoculation and B2R mRNA increased in the skin at 3 weeks. Recently we reported that B2R mRNA is increased in DRGs, and the B2R-mediated sensitivity of C-fiber nociceptors to bradykinin is increased in inflamed animals.[7] However, it is not known whether increased B2R mRNA in DRGs contributes to increased B2R mRNA in the skin. Reportedly, VR1 mRNA is transported through axons to the periphery in inflamed conditions; therefore, B2R mRNA may also be transported to the periphery in the nerves,[15] and may contribute to increase in B2R mRNA in the skin. Perfusion of the skin samples did not change mRNA levels, which suggests that B2R mRNA is expressed in the resident cells in the skin.

Both B1R and B2R mRNA increased in the skin of inflamed animals. The increase was statistically significant at three weeks after inoculation. The latency of these increases is longer than previous findings for B2R mRNA increases in

DRGs,[16] and longer than the time at which the signs of inflammation become apparent at 2 weeks after inoculation. NGF is known to play an important role in the mechanism of increasing B2R expression in DRGs.[17-20] For example, the content of NGF, which is believed to be produced in the peripheral tissues and transported to DRGs, increased in DRGs at 2 days after CFA inoculation and reached a maximum 2 weeks after inoculation (unpublished observation of our laboratory). This result may suggest that the regulation of both B1R and B2R mRNA expression in the skin is different from that of B2R in DRGs.

The present result showed that B1R mRNA is expressed in normal skin. It has been thought that B1R is usually not expressed and does not participates in pain/hyperalgesia in normal animals, but is synthesized *de novo* following tissue injury and inflammation[5] to play an important role in pain/hyperalgesia. It would be reasonable to hypothesize that B1R mRNA is not translated in the skin, or that B1R normally exists in an inactive form and it is activated only in a state of inflammation. Perfusion of the skin samples from animals at 2 weeks after inoculation did not change the expression of B1R mRNA. Therefore, the possibility is high that mRNA is expressed in resident cells of the skin. Our present experiment does not provide any data as to which kind of cells express B1R mRNA. It is thought that B1R mRNA is expressed in cells other than neurons, such as epithelial cells and fibroblasts, and exerts its hyperalgesic action through the release of some substances that activate nociceptors from these cells.

In the present study, B1R mRNA was detected in the skin of the control animals and increased in inflammation. In addition to these findings, previous studies showed that B1R antagonist had an analgesic action only in inflamed conditions. These two facts lead us to the hypothesis that B1R mRNA is translated or B1R is activated only in tissue in an inflammatory state.[21] However, the mechanism for this activation is unknown and remains open to future study.

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