Potentiation of transient receptor potential V1 functions by the activation of metabotropic 5-HT receptors in rat primary sensory neurons

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5-Hydroxytryptamine (5-HT) is one of the major chemical mediators released in injured and inflamed tissue and is capable of inducing hyperalgesia *in vivo*. However, the cellular mechanisms of 5-HT-induced hyperalgesia remain unclear. Transient receptor potential V1 (TRPV1) plays a pivotal role in nociceptive receptors. In the present study, we determined whether 5-HT changes TRPV1 functions in cultured dorsal root ganglion (DRG) neurons isolated from neonatal rats, using Ca^{2+} imaging and whole-cell patch-clamp techniques. In more than 70% of DRG neurons, 5-HT potentiated the increases of $[Ca^{2+}]_i$ induced by capsaicin, protons and noxious heat. Capsaicin-induced current and depolarizing responses, and proton-induced currents were also augmented by 5-HT. RT-PCR analysis revealed the expression of 5-HT_{2A} and 5-HT_7 receptors in rat DRG neurons. Agonists for 5-HT_{2A} and 5-HT_7 receptors mimicked the potentiating effect of 5-HT, and their antagonists decreased it. In DRG ipsilateral to the complete Freund's adjuvant-injected inflammation side, expression levels of 5-HT_{2A} and 5-HT_7 mRNAs increased, and the potentiating effect of 5-HT was more prominent than in the contralateral control side. These results suggest that the PKC- and PKA-mediated signalling pathways are involved in the potentiating effect of 5-HT on TRPV1 functions through the activation of 5-HT_{2A} and 5-HT_7 receptors, respectively. Under inflammatory conditions, the increases of the biosynthesis of these 5-HT receptors may lead to further potentiation of TRPV1 functions, resulting in the generation of inflammatory hyperalgesia *in vivo*.

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Tissue damage associated with inflammation produces an array of chemical mediators that sensitize nociceptor terminals to elicit exacerbate pain. A number of substances released during inflammation such as bradykinin (BK), prostaglandin E_2 (PGE_2) and 5-hydroxytryptamine (5-HT) generate pain and hyperalgesia (Kress & Reeh, 1996).

Transient receptor potential V1 receptor (TRPV1) is a non-selective cation channel gated by capsaicin, noxious heat and protons (Caterina *et al.* 1997; Tominaga *et al.* 1998). TRPV1 function is regulated by a variety of inflammatory mediators. BK reduces the heat threshold of TRPV1 through protein kinase C (PKC) activation (Cesare & McNaughton, 1996; Sugiura *et al.* 2002). Nerve growth factor and ATP sensitize TRPV1 in a PKC-dependent manner (Bonnington & McNaughton, 2003; Moriyama *et al.* 2003). PGE_2 potentiates TRPV1-mediated responses via a cyclic AMP/protein kinase A (cAMP/PKA) pathway in rat sensory neurons (Lopshire & Nicol, 1998; Moriyama *et al.* 2005). In mice lacking TRPV1, heat hyperalgesia fails to occur during inflammation (Caterina *et al.* 2000; Davis *et al.* 2000), suggesting that TRPV1 is critical for inflammatory heat hypersensitivity.

5-Hydroxytryptamine is released primarily from descending bulbospinal serotonergic neurons and causes analgesia (Sorkin *et al.* 1993; Liu *et al.* 2002). In the periphery, 5-HT is released from platelets, mast cells and endothelial cells into a wound site in response to inflammation and injury (Lehtosalo *et al.* 1984). This chemical is a potent pro-inflammatory and pro-nociceptive agent since it excites nociceptive afferents (Beck & Handwerker, 1974) and induces hyperalgesia in humans (Ernberg *et al.* 2000; Schmelz *et al.* 2003) and rats (Sufka *et al.* 1992; Taiwo & Levine, 1992). 5-HT receptors are classified into 7 families and 13 subtypes (Hoyer *et al.* 2002). It is recognized that ionotropic 5-HT_3 receptors are

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located on sensory nerve terminals and are responsible for 5-HT-induced pain and hyperalgesia (Eschalier *et al.* 1989; Giordano & Rogers, 1989). However, it is also reported that other subtypes of 5-HT receptors participate in inflammatory pain (Ebersberger *et al.* 1995; Abbott *et al.* 1996; Espejo & Gill, 1998; Tokunaga *et al.* 1998; Okamoto *et al.* 2002). Therefore, despite the potential importance of 5-HT in hyperalgesia, the involvement of 5-HT receptor subtypes in hyperalgesia and cellular mechanisms remained to be clarified.

Recently, some reports have suggested the possibility that the hyperalgesic action of 5-HT is related to TRPV1 functions. Stimulation of spinal 5-HT receptors potentiates the capsaicin-induced release of substance P in the rat dorsal horn *in vivo* (Bertelsen *et al.* 2003), and 5-HT enhances TRPV1 function in mouse colon sensory neurons (Sugiura *et al.* 2004).

The aim of the present experiment was to identify the cellular mechanisms and related receptor subtypes in 5-HT-induced hyperalgesia responsible for TRPV1 functions in dorsal root ganglion (DRG) neurons *in vitro*. As TRPV1 possess high Ca^{2+} permeability (Caterina & Julius, 2001), it is possible to monitor their functions by measuring the $[Ca^{2+}]_i$ response in individual DRG neurons systematically. In the major part of the present study, therefore, we used a Ca^{2+}-imaging technique to evaluate effects of 5-HT on TRPV1-mediated responses. 5-HT-induced changes of membrane potential and current responses to capsaicin were also investigated. 5-HT receptor subtypes and related intracellular signalling pathways were determined by pharmacological and molecular analyses. Finally, we tested whether 5-HT receptor expression and the actions of 5-HT were changed under inflammatory conditions.

Methods

Isolation and culture of DRG neurons

All protocols for the use of animals were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University. Neonatal Wistar rats (1–4 days old) of either sex were decapitated under ethyl ether anaesthesia. The spinal column was opened and DRGs were detached under a dissecting microscope. The DRGs were trimmed of attached fibres in Ca^{2+},Mg^{2+}-free phosphate-buffered saline (PBS (mM): 136.9 NaCl, 2.7 KCl, 1.47 KH_2PO_4, 8.1 Na_2HPO_4), enzymatically digested for 30 min in PBS-containing collagenase (1 mg ml^{-1}, Type I, Worthington, Lakewood, NJ, USA) and DNase I (0.5 mg ml^{-1}, Roche, Indianapolis, IN, USA), and then for another 30 min in PBS-containing trypsin (0.25% w/v, Type XI, Sigma, St Louis, MO, USA) and DNase I at 37°C. Subsequently, they were rinsed with culture medium, M199 (Sigma) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 μg ml^{-1}, Bunyu, Japan) and streptomycin (100 u ml^{-1}, Meiji-Seika, Japan). Following enzyme digestion, individual cells were mechanically dispersed by repeated triturations using a fire-polished Pasteur pipette and stood on ice. Then the cell suspension was centrifuged (100 *g*, 5 min, 4°C) and the pellet containing cells was resuspended with the culture medium. Aliquots were placed onto glass coverslips coated with poly D-lysine (Sigma). Cells were kept in the culture medium without nerve growth factor (NGF) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. DRG neurons were used between 12 and 36 h after the plating. In the experiments using DRG isolated from inflammation-induced rats, fresh uncultured DRG neurons were used.

Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in single cells was measured with a fluorescent Ca^{2+} indicator, fura-2, by dual excitation using a fluorescence imaging system controlling illumination and acquisition with software (Aqua Cosmos, Hamamatsu Photonics, Japan) as previously described (Ohta *et al.* 2005*b*). To load fura-2, cells were incubated for 1 h at room temperature with a 10 μM concentration of the acetoxymethyl ester form of fura-2 (fura-2 AM; Molecular Probes, Eugene, OR, USA) in normal external solution (mM): 134 NaCl, 6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, 10 Hepes (pH 7.4 with NaOH). A coverslip with fura-2-loaded cells was placed in an experimental chamber mounted on the stage of an inverted microscope (Diaphot 300, Nikon, Japan). In some experiments, before $[Ca^{2+}]_i$ measurement, cells were incubated with the plant lectin, isolectin GS-IB4 from *Griffonia simplicifolia* Alexa Fluor 488 conjugate (IB4; 10 μg ml^{-1}, Molecular Probes) for 10 min and then rinsed for 2 min. IB4 staining was visualized with appropriate filters (excitation 490 nm; emission barrier filter 515–555 nm). A neuron was considered IB4-positive if it had a continuous green ring around the perimeter. Cells were illuminated every 2 s with lights at 340 and 380 nm and the respective fluorescence signals (F_{340} and F_{380}) were detected. The fluorescence emitted was projected onto a CCD camera, and F_{340}, F_{380} and its ratio (F_{340}/F_{380}) were stored on the hard disk of a computer (Pro-600 l, Epson, Japan). Since fluorescence signals of fura-2 are influenced by changes in pH and temperature, we adopted data from cells of which the F_{340} and F_{380} showed a mirror image. Calibration of fura-2 was performed with a Ca^{2+} calibration buffer solution (Molecular Probes) containing 5 μM fura-2 (Ohta *et al.* 2005*b*). Cells were continuously superfused with the external solution through multibarrelled tubes by

Table 1. 5-HT receptor and β-actin primers for RT-PCR

Name	Sense (upper row)/ antisense (lower row)	Product size (bp)	Accession no.
5-HT_{1A}	5′-TCACCTGCGACCTGTTTATC-3′	394	J05276
	5′-GCTCCCTTCTTTTCCACCTT-3′		
5-HT_{1B}	5′-TCGTGCTGGTGTGGGTCTTCT-3′	262	X62944
	5′-ATCAACTGGGCTCGGGTCAAG-3′		
5-HT_{1D}	5′-CTGAATGCTACAGGGGCTTGG-3′	452	M89953
	5′-CGGTGGGATGGAGATACAGA-3′		
5-HT_{1F}	5′-ACCTTTGGCTGAGTGTTGAC-3′	591	L05596
	5′-TAGTGGCTGCTTTGCGTTCT-3′		
5-HT_{2A}	5′-GCATCGAACTGGACAATTGATGCTGAAAA-3′	611	M30705
	5′-ATGAAAAATGCCACAAAAGAGCCTATGAG-3′		
5-HT_{2B}	5′-GTGATGCCGATTGCTCTCTT-3′	297	X66842
	5′-GATGTTGTGTGCGTTGACCA-3′		
5-HT_{2C}	5′-TTCGTTCTCATCGGGTCCTT-3′	441	U35315
	5′-CACATAGCCAATCCACACAA-3′		
5-HT_{3}	5′-GAACTACAAGCCCCTACAGC-3′	439	D49395
	5′-TGACACGATGATGAGAAAGA-3′		
5-HT_{4}	5′-GGGAGATGTTTTGCCTGGTC-3′	484	U20907
	5′-CGATGTGTGCTGTGCTGGTC-3′		
5-HT_{5A}	5′-TTCCACCGAGTACCACACAA-3′	757	L10072
	5′-TGACGGACAGTGAACACCAT-3′		
5-HT_{5B}	5′-ACGTGTGGATCTCCTTCGAC-3′	426	L10073
	5′-CAGACTCCTGAGGTGCTTCC-3′		
5-HT_{6}	5′-TACTGTAATAGCACCATGAACCCTATCAT-3′	374	L41146
	5′-CTGAGTGGATGCGGCCGTATCTCAGGCTC-3′		
5-HT_{7}	5′-AGCCCTCCAACTACCTGATT-3′	566	L22558
	5′-ACACTCTTCCACCTCCTTCT-3′		
β-Actin	5′-AGCCATGTACGTAGCCATCC-3′	294	V01217
	5′-GCCATCTCTTGCTCGAAGTC-3′		

gravity at a flow rate of $\sim$1 ml min^{-1}, and the bath level was adjusted so that the total bath volume was about 0.5 ml. Drugs were applied through different tubes of puffer pipettes. All experiments were carried out at room temperature (20–24°C) except the experiment on heat effects. Noxious heat stimulation was carried out by the application to the cells of an excess volume of external solution heated to the desired temperature, as reported previously (Ohta *et al.* 2005*a*).

Whole-cell membrane voltage and current recordings

Membrane currents and potentials were recorded using the conventional whole-cell configuration of the patch-clamp technique (Ohta *et al.* 2001). Whole-cell recordings were made from small diameter neurons ($< 20\ \mu$m) using glass electrodes with 4–5 MΩ resistance mounted on the head stage of a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA, USA). Current data were filtered at 1 kHz and sampled at 2–5 kHz by an A/D converter (PowerLab System, ADInstruments, Australia) in conjunction with a personal computer (Macintosh G3, Apple, Japan), and stored on the hard disk of the PC. The cell capacitance of DRG neurons was 15.8 ± 0.4 pF ($n = 92$). After cells were voltage clamped at a holding potential of −60 mV, a step depolarization to 0 mV lasting 50 ms was applied for the activation of voltage-dependent Na^+ channels to confirm that they were DRG neurons. The standard pipette solution contained (mM): 130 CsCl, 6 NaCl, 4 Mg-ATP, 0.3 $GTPNa_3$, 10 Hepes and 10 EGTA (pH 7.3 with CsOH). For current-clamp study, K^+-based pipette solution was used (mM: 130 potassium gluconate, 6 NaCl, 4 Mg-ATP, 0.3 $GTPNa_3$, 10 Hepes and 0.2 EGTA, pH 7.3 with KOH). A multibarrelled puffer system similar to that used for $[Ca^{2+}]_i$ measurement was used for drug application and external perfusion.

Reverse transcription-polymerase chain reaction

The design of the oligonucleotides used for the specific amplification of rat 5-HT receptor cDNAs was based on sequences registered in GenBank for each of the receptors in the rat. The nucleotide sequence and the length of the expected PCR product for each primer pair are shown in Table 1. Total RNA from rat DRG was extracted with Trizol reagent (Isogen, Nippon Gene, Japan), and then treated with DNase I (Promega, Madison, WI, USA). First-strand cDNA was synthesized from oligo(dT)-primed total RNA

with Superscript II reverse transcriptase (Gibco). The reaction mixture was then subjected to PCR amplification with the use of *Taq* DNA polymerase (Promega). Samples were heated to 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. To exclude the possibility of genomic DNA giving a false-positive result in the PCR, a reverse transcriptase-free (negative) control was run with each primer pair. The PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining followed by UV transillumination. We identified the respective subtypes of receptors by extracting them from gel, subcloning with pGEM-T-easy vector (Promega) and sequencing (CEQ8000, Beckman, CA, USA) (Ohta *et al.* 2005*a*).

Complete Freund's adjuvant-induced inflammation

Neonatal rats were injected with complete Freund's adjuvant (CFA, 50 μl; Sigma) into the plantar surface of the right hind paw (Ruda *et al.* 2000). For the control, the same amount of saline was injected into the left hind paw. Injection of this dose of CFA caused inflammation of the entire hind leg of the rat compared with the control leg (see Fig. 6*A*). As reported by Ruda *et al.* (2000), the rats exhibited distinct behavioural responses identical to those observed in an adult model of pain (Abbott *et al.* 1995). Four days after CFA injection, both inflamed and control sides of DRG at L4–L6, which innervate the hind legs, were separately removed, and enzymatically isolated for the following $[Ca^{2+}]_i$ measurement, and mRNA was extracted for the following RT-PCR analysis. For histological observation, dissected legs were fixed with formalin and cross-sections were cut at the base of the leg after paraffin embedding and stained with haematoxylin–eosin by a standard protocol. A total of 12 rats were used for evoking CFA-induced leg inflammation.

Materials

The following drugs were used: bisindoylmaleimide I (BIM), capsaicin, (±)-2,5-dimethoxy-4-iodoamphetamine (DOI), *N*-2-hydroxyethyl-piperazine-2-ethanone-sulphonic acid (Hepes) (±)-8-hydroxy-2(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT), 5-hydroxytryptamine (5-HT), ketanserin and SB469970 ((*R*)-3-(2-(2-(4-methypiperidin-1-yl)ethyl-)-pyrrolidine-1-sulphonyl-phenol) from Sigma. *N*-[2-(*p*-bromo-cinnamyl-amino)ethyl]-5-isoquinoline-sulphonamide (H89) was from Seikagaku-Kogyo (Japan). All other drugs used were of analytical grade.

Data analysis

The data are presented as the mean ± S.E.M. (n = number of cells, N = number of animals). The present data were obtained from at least two different coverslips for each different culture (animal) per experiment. Comparisons were made by Student's paired t test when comparing responses before and after the application of test drugs or by one-way ANOVA followed by the Tukey-Kramer test when more than two groups were compared (StatView 5.0, SAS Institute Inc., USA). Differences with a P value of less than 0.05 were considered significant. The EC_{50} value was determined using Origin software (OriginLab, USA).

Results

5-HT potentiates TRPV1-mediated responses

To assess the possibility that 5-HT modulates TRPV1 functions, we examined the effects of 5-HT on the capsaicin-induced $[Ca^{2+}]_i$ increase. Approximately 75% of neonatal rat DRG neurons could respond to 1 μM capsaicin (data not shown). DRG neurons were repetitively stimulated with capsaicin (30 nM) for 15 s with an interval of 8 min. 5-HT was applied 3 min prior to and during the second application of capsaicin. Figure 1*A* shows two representative $[Ca^{2+}]_i$ responses to capsaicin and the effect of 5-HT on them. The $[Ca^{2+}]_i$ increase induced by capsaicin in the presence of 5-HT (S2) significantly increased in comparison with that in its absence (S1) (Fig. 1*B*). In a few neurons (6.5%, 15 of 231 cells), a transient $[Ca^{2+}]_i$ increase was elicited by 5-HT (Fig. 1*Ab*). Regardless of whether a 5-HT-induced $[Ca^{2+}]_i$ increase occurred, $[Ca^{2+}]_i$ responses to capsaicin were augmented by 5-HT. The ratio (S2/S1) of the $[Ca^{2+}]_i$ increases before to that after exposure to 5-HT was 2.4 ± 0.3 times. Because the extent of the potentiating effect varied from cell to cell, we determined the ratio (S2/S1) from individual cells, and the percentage of neurons was plotted against each ratio value with and without exposure to 5-HT (Fig. 1*C*). In control cells (without 5-HT), a large number of cells were distributed below the ratio of 1.5. In the 5-HT treatment group, the proportion of cells showing a ratio of over 2 increased to 75.3%, whereas it was only 6.6% in the control. The histogram showing the percentage of cells–ratio (S2/S1) relation shifted to the right.

It has been reported that nociceptors can be divided into two main groups; one class expresses trkA receptors and contains neuropeptides such as calcitonin gene-related peptide and substance P, and the other depends on glial cell line-derived neurotrophic factor (GDNF) and contains few neuropeptides but binds isolectin IB4 (Molliver *et al.* 1997; Bennett *et al.* 1998). Differential response properties of IB4-positive and -negative sensory neurons have been reported (Dirajlal *et al.* 2003; Liu *et al.* 2004; Breese *et al.* 2005). In the present results, however, there was no clear difference in the distribution of the histograms between IB4-positive and -negative neurons (Fig. 1*D*).

Using the whole-cell patch-clamp technique, the effects of 5-HT on the membrane potential change and inward current in response to capsaicin were investigated. Under current-clamp conditions, the resting membrane potential was -53.9 ± 0.7 and capsaicin (30 nM) depolarized membrane potentials (Fig. 2*A*). The resting membrane potential did not change in the presence of 10 μM 5-HT (-51.5 ± 0.8 mV, $n = 24$). After the application of 5-HT, the depolarization induced by capsaicin was clearly increased in 9 of 24 cells, and in 3 cells prominent spike discharges were superimposed on the sustained depolarization (Fig. 2*A*). Summarized changes in capsaicin-induced depolarization in the absence and the presence of 5-HT are shown in Fig. 2*C*. Under voltage-clamp conditions, capsaicin evoked an inward current (8.1 ± 1.9 pA pF^{-1}, $n = 39$) at a holding potential of -60 mV. An obvious augmentation of capsaicin-induced current by 5-HT was observed in 12 of 39 cells. The peak amplitude of capsaicin-induced inward current was significantly increased in the presence of 5-HT (Fig. 2*D*).

TRPV1 is known as a polymodal receptor, that is activated not only by the vanilloid agonist capsaicin, but also by protons and noxious heat (Caterina *et al.* 1997; Tominaga *et al.* 1998). We next examined whether 5-HT produced potentiating effects on the proton- or

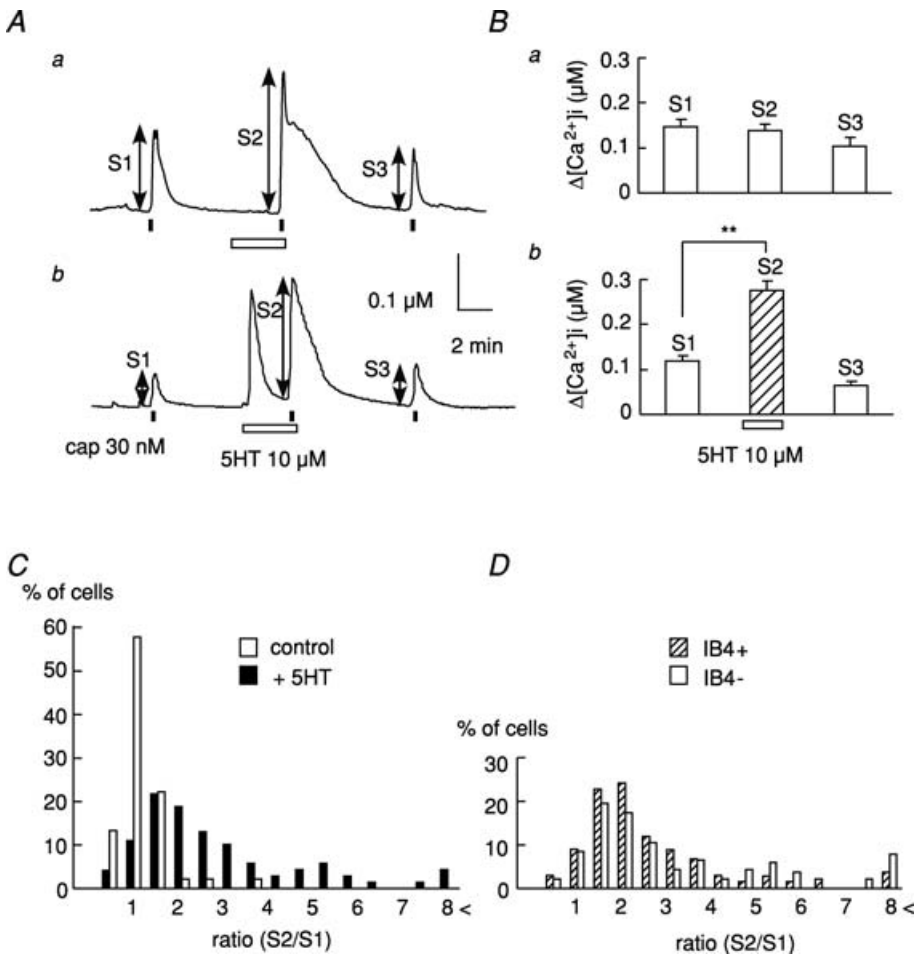

Figure 1. Potentiating effects of 5-HT on the $[Ca^{2+}]_i$ responses to capsaicin
A, representative $[Ca^{2+}]_i$ increases induced by capsaicin (cap, 30 nM) before, during the application of 5-HT (10 μM), and after its removal. 5-HT failed to produce a $[Ca^{2+}]_i$ increase in the majority of cells (*a*), but in some cells, 5-HT elicited a $[Ca^{2+}]_i$ increase (*b*). *B*, summarized data on changes in the $[Ca^{2+}]_i$ increase induced by capsaicin before (S1), without (*a*) or with (*b*) 5-HT (S2) and after its washout of 5-HT (S3). The increment of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) shown by the double-ended arrows was plotted (control, $n = 89$, $N = 4$; +5-HT, $n = 231$, $N = 5$). ** $P < 0.01$. *C*, histogram showing the percentage of cells *versus* the ratio (S2/S1) of the capsaicin-induced $[Ca^{2+}]_i$ increase. In the control, data were obtained from the 3 repeated applications of capsaicin without 5-HT. *D*, histogram showing the percentage of cells–S2/S1 ratio relation for IB4-positive (IB4+; $n = 129$, $N = 5$) and -negative neurons (IB4−; $n = 102$, $N = 5$) in the presence of 5-HT. These data were transcribed from *C* (+5-HT) after sorting cells by the differences in IB4 staining.

heat-induced $[Ca^{2+}]_i$ responses. As shown in Fig. 3*A*, a brief application of protons (pH 5.5) or heat (48°C) produced a transient $[Ca^{2+}]_i$ increase. After the application of 5-HT (10 μM), each $[Ca^{2+}]_i$ response was significantly enhanced (Fig. 3*B*). Similar to the responses to capsaicin, these potentiating responses occurred in both IB4-positive and -negative neurons (Fig. 3*C*).

The effects of 5-HT on proton-induced currents at a holding potential of −60 mV were also examined (Fig. 3*D*). An application of protons (pH 5.5) elicited three distinct inward currents in neonatal rat DRG neurons: a transient current with a rapid inactivation (3 of 25 cells), a transient current followed by a sustained current (12 of 25 cells, shown in Fig. 3*D*) and only a sustained current (10 of 25 cells). Similar current patterns have been reported in adult rat DRG neurons (Liu *et al.* 2004). In the presence of 5-HT (10 μM), a sustained component of inward current evoked by protons was significantly augmented. These results indicate that 5-HT potentiates TRPV1 functions in rat DRG neurons.

Involvement of PKC and PKA in the potentiating effect of 5-HT on TRPV1

It is known that either BK or PGE_2 sensitizes TRPV1 functions in adult rat DRG neurons through the activation of PKC (Cesare & McNaughton, 1996; Cesare *et al.* 1999; Premkumar & Ahern, 2000) and cAMP/PKA pathways (Lopshire & Nicol, 1998; Gu *et al.* 2003; Moriyama *et al.* 2003), respectively. To investigate the mechanism underlying 5-HT-induced augmentations of TRPV functions, we examined the effects of bisindoylmaleimide I (BIM, 0.5 μM), a PKC inhibitor, and H89 (1 μM), a PKA inhibitor, on the potentiating action of 5-HT. As shown in Fig. 4*A*, each inhibitor was applied for 4 min before 5-HT treatment to the end of the second application of capsaicin. Since the extent of the 5-HT-induced potentiating effect was different among neurons (Fig. 1*C*), the effects of these kinase inhibitors were estimated by using the data from cells in which clear potentiation by 5-HT occurred after the removal of these inhibitors. Figure 4*B* depicts summarized data showing that these inhibitors suppressed 5-HT-induced potentiation of $[Ca^{2+}]_i$ responses to capsaicin (S1 *versus* S2). Figure 4*C* shows histograms of the percentage of cells with the indicated ratio of capsaicin-induced responses in the presence (S2/S1) and after the removal (S4/S3) of BIM or H89. The population of neurons shows that the ratio (>1.5) was reduced from 73.3% to 30.9% by BIM and from 77.6% to 36.2% by H89, and the percentage of cells–ratio relation was shifted leftward. However, even in the presence of these inhibitors, there were neurons showing significant potentiation to an extent almost equal to that after the removal of inhibitors (21 of 84 neurons for BIM, 15 of 58 cells for H89). Moreover, BIM seemed to reduce responses to capsaicin, because 22.8% of cells *demonstrated* a ratio <0.5 in the presence of BIM but not H89. Taken together, these results suggest that both PKC and PKA pathways are involved in the potentiating effect of 5-HT on TRPV1 functions in rat neonatal DRG neurons.

5-HT receptor subtypes in rat DRG neurons

To identify the subtypes of 5-HT receptors expressed in the rat DRG, RT-PCR using 13 pairs of primers for 5-HT receptors was performed. The major PCR products

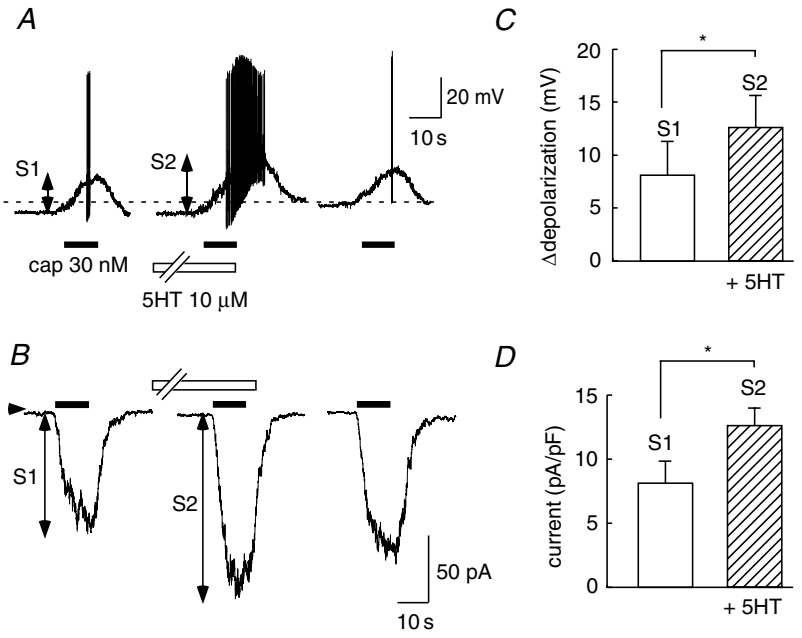

Figure 2. 5-HT potentiates depolarization and the inward current induced by capsaicin
A, under current-clamp conditions, capsaicin (30 nM) was repetitively applied before and during the application of 5-HT (10 μM), and after its washout. The dashed line indicates the −40 mV level. In this cell, 5-HT increased capsaicin-induced depolarization concomitant with a marked spike discharge. *B*, at a holding potential of −60 mV, capsaicin evoked inward currents that were augmented by 5-HT. Change in amplitude of membrane potentials (*C*, $n = 24$, $N = 4$) and inward currents (*D*, $n = 39$, $N = 5$) induced by capsaicin before (S1) and after the application of 5-HT (S2). * $P < 0.05$.

detected in rat DRG were 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_3, 5-HT_4, 5-HT_{5B} and 5-HT_7 (Fig. 5*A*). Based on the examination of intracellular signalling, PKC and PKA systems were suggested to play a role in the potentiating effects of 5-HT on TRPV1. Among the 5-HT receptors detected by PCR, 5-HT_{2A} and 5-HT_7 are coupled to $G_{q/11}$-PLC/PKC and G_s-adenylate cyclase (AC)/PKA pathways, respectively (Hoyer *et al.* 2002; Alexander *et al.* 2006). Therefore, to examine the possibility that 5-HT_{2A} and 5-HT_7 receptors were involved in the potentiating

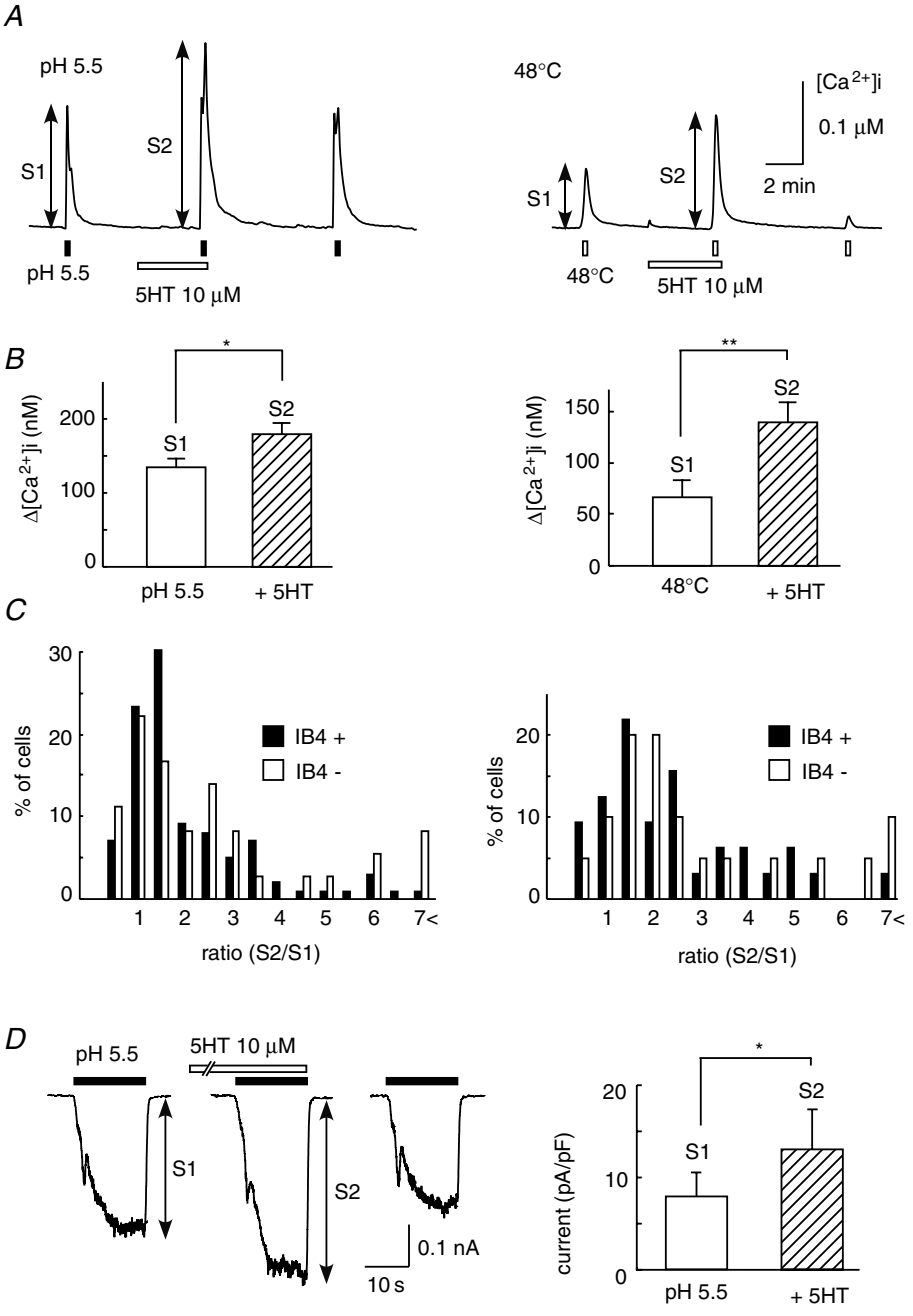

Figure 3. Enhancement of $[Ca^{2+}]_i$ responses to protons and noxious heat by 5-HT
A, representative $[Ca^{2+}]_i$ responses to protons (pH 5.5, left) and noxious heat (48°C, right) before and during the application of 5-HT (10 μM), and after its removal. *B*, summarized changes in $[Ca^{2+}]_i$ induced by protons (left, $n = 119$, $N = 4$) and noxious heat (right, $n = 77$, $N = 4$), before (S1) and after the application of 5-HT (S2). * $P < 0.05$, ** $P < 0.01$. *C*, histograms of the percentage of cells–S2/S1 ratio relation obtained by stimulation with protons (left) and noxious heat (right), between IB4-positive (IB4+) and -negative neurons (IB4−) in the presence of 5-HT. *D*, representative currents induced by protons (pH 5.5, 15 s) at −60 mV before (S1), during the application of 5-HT (10 μM, S2) and after its removal, and summarized results ($n = 25$, $N = 4$). * $P < 0.05$.

effects of 5-HT, we first carried out a pharmacological study using selective agonists and antagonists. A protocol similar to that shown in Fig. 1*A* was used to investigate whether agonists to 5-HT_{2A} and 5-HT_7 receptors produced potentiating effects on TRPV1 function. Changes in the $[Ca^{2+}]_i$ increase in response to capsaicin before and after treatment with (±)-2,5-dimethoxy-4-iodoamphetamine (DOI, 20 μM), a 5-HT_{2A} agonist, or (±)-8-hydroxy-2(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT, 20 μM), a 5-HT_7 agonist, are summarized in Fig. 5*B*. Both agonists elicited significant potentiation of the capsaicin-induced $[Ca^{2+}]_i$ increase.

Next, we examined the effects of 5-HT_{2A} and 5-HT_7 antagonists on 5-HT-induced potentiation of responses to capsaicin. For these experiments, we used a protocol similar to that shown in Fig. 4*A*. Figure 5*C* depicts

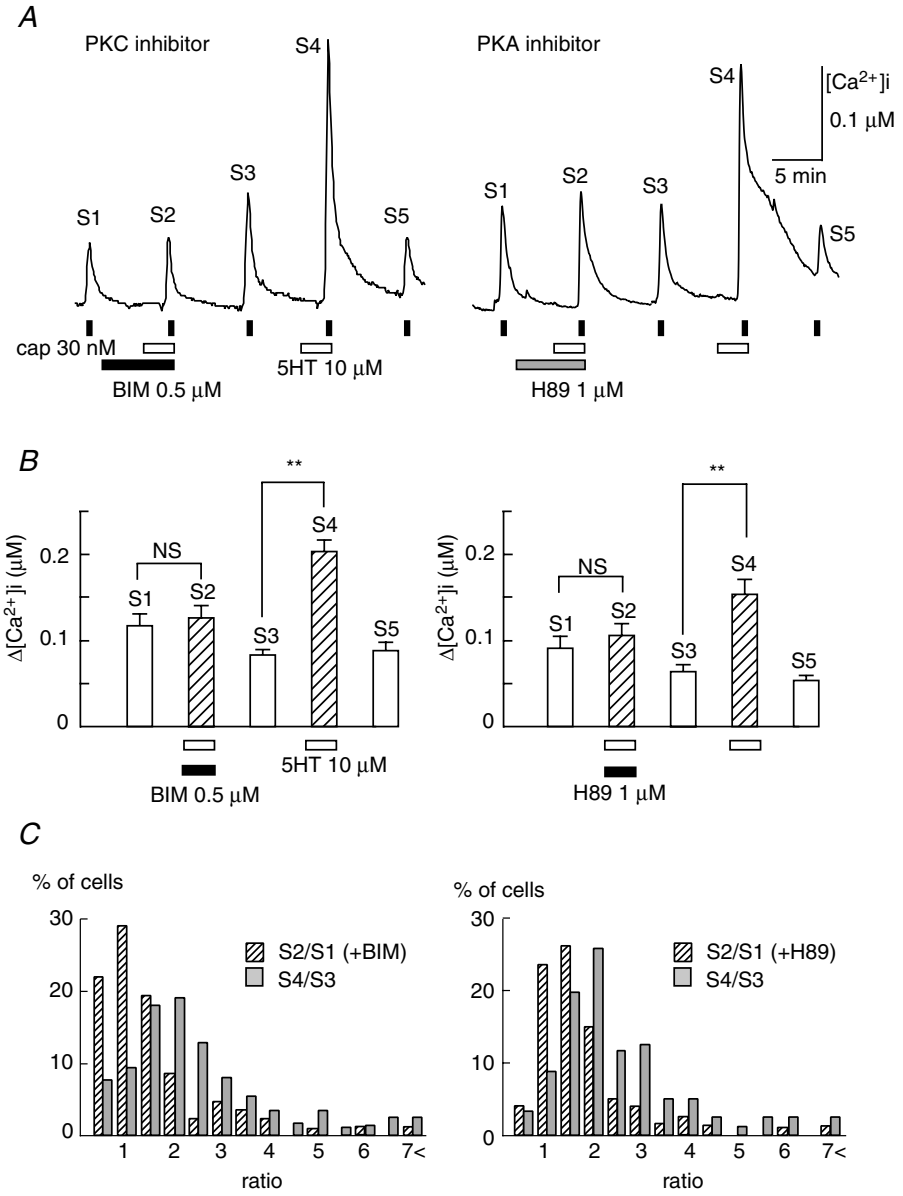

Figure 4. Effects of inhibitors of PKC and PKA on capsaicin-induced $[Ca^{2+}]_i$ increases
A, representative $[Ca^{2+}]_i$ increases induced by capsaicin (cap, 30 nM) in the presence and absence of a kinase inhibitor and/or 5-HT (10 μM). Bisindoylmaleimide I (BIM, 0.5 μM), a PKC inhibitor, and H89 (1 μM), a PKA inhibitor, were used. Cells were stimulated by capsaicin 5 times. The second stimulation was carried out in the presence of 5-HT with a kinase inhibitor and the fifth stimulation in the presence of 5-HT without it. The changes in $[Ca^{2+}]_i$ in response to each application of capsaicin, shown as S1–S5, are summarized in *B*. BIM, $n = 84$, $N = 5$; H89, $n = 58$, $N = 4$; NS, not significant, ** $P < 0.01$. *C*, histograms of the percentage of cells–ratio relations; data obtained in the presence and absence of a kinase inhibitor (S2/S1) and after its washout (S4/S3).

summarized data in the presence and absence of ketanserin (20 μM), a 5-HT_{2A} antagonist, and SB269970 (20 μM), a 5-HT_7 antagonist. After exposure to these antagonists, the sensitizing effects of 5-HT were significantly suppressed. The population of neurons showing a ratio > 1.5 was reduced from 78.9% to 48.3% for ketanserin and 74.4% to 36.4% for SB269970. However, even in the presence of each inhibitor there were neurons showing potentiation to the same extent as that after the removal of the inhibitor (8 of 38 cells for ketanserin, 6 of 46 cells for SB269970). These results suggest that 5-HT_{2A} and 5-HT_7 receptors are partly involved in the TRPV1 potentiation by 5-HT in rat neonatal DRG neurons.

Change of 5-HT action under inflammatory conditions

To examine the role of 5-HT in the hyperalgesia following peripheral inflammation, we investigated whether the sensitizing effects of 5-HT were changed by inflammation. Inflammatory model animals were made by the injection of complete Freund's adjuvant (CFA) into the right hemilateral hind paw of the neonatal rat. The same volume of saline was injected into the left hemilateral hind paw as a control. Four days after CFA administration, the limb in the CFA-administrated side was prominently swollen in macroscopic observation (Fig. 6*A*). Histological analysis demonstrated typical inflammation images with the accumulation and invasion of leucocytes in the inflammatory side compared with the control side (Fig. 6*B*). We isolated DRG of the L4–L6 segment, which innervates the hind limb area, synthesized cDNA, and then performed RT-PCR analysis. As shown in Fig. 6*C*, mRNA expression of 5-HT_{2A} and 5-HT_7, but not 5-HT_{1D}, increased in DRG of the inflamed side. Similar changes in 5-HT receptor expression were observed in four other animals. Quantitative analysis for these PCR products showed significant increases of 5-HT_{2A} and 5-HT_7 receptor mRNAs in DRG of the inflammation site (Fig. 6*D*).

To determine whether functional changes were accompanied by increases in mRNA expression, DRG from both sides were isolated from rats injected with CFA and the potentiating effects of 5-HT were compared. In these experiments, acute isolated DRG neurons were used. A protocol similar to that shown in Fig. 1*A* was used to estimate possible changes in the TRPV1 potentiation effects of 5-HT. As shown in Fig. 6*E*, the ratio of potentiation (S2/S1) in DRG neurons isolated from the inflammation side was significantly larger than that from the control side. The histograms of the percentage of cells–ratio relation in DRG neurons of inflammation and control sides are shown in Fig. 6*F*. There was no significant difference in the magnitude of the capsaicin-induced $[Ca^{2+}]_i$ increase in DRG neurons

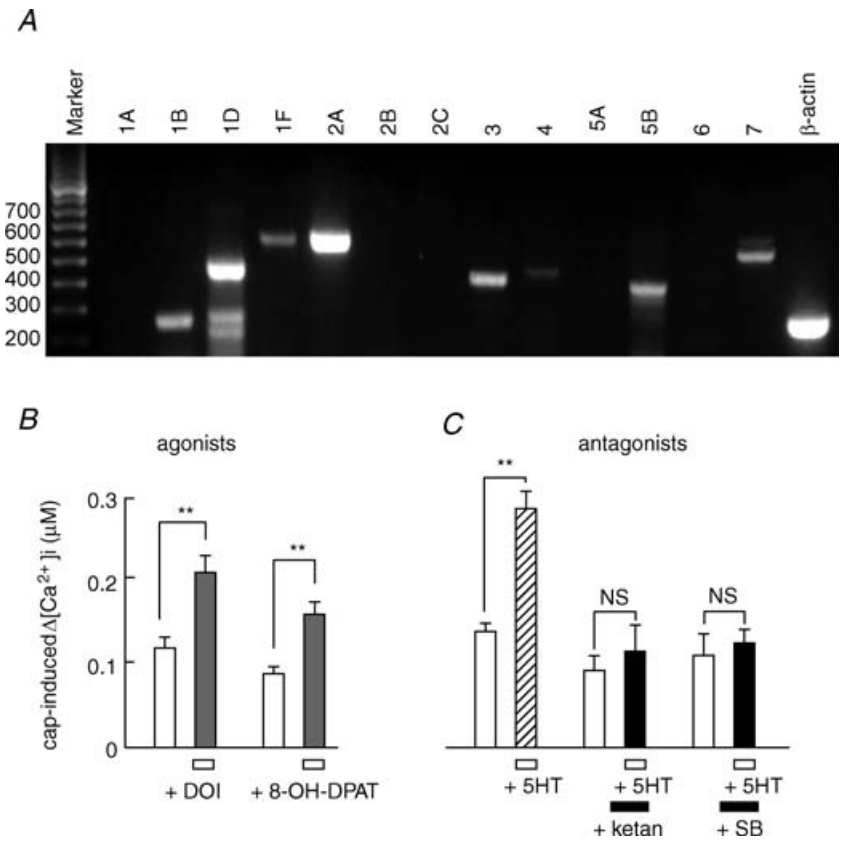

Figure 5. Involvement of 5-HT_{2A} and 5-HT_7 receptors in the potentiating effects of 5-HT
A, RT-PCR analysis of 5-HT receptor mRNA. Specific mRNA primers for each 5-HT receptor and β-actin were used for RT-PCR analysis of cDNA synthesis from total RNA of neonatal rat DRG. The expected size of mRNA for each subtype was detected by each primer. Size markers (bp) are shown on the left. *B*, effects of 5-HT_{2A} and 5-HT_7 agonists on the capsaicin-induced $[Ca^{2+}]_i$ increases. DOI (20 μM, $n = 20$, $N = 3$), a 5-HT_{2A} agonist and 8-OH-DPAT (20 μM, $n = 52$, $N = 4$), a 5-HT_7 agonist, were used. The panel shows summarized $[Ca^{2+}]_i$ increases induced by capsaicin (30 nM) before and during the application of each 5-HT agonist. *C*, inhibitory effects of 5-HT antagonists on 5-HT-induced potentiation of $[Ca^{2+}]_i$ responses to capsaicin. Ketanserin (+ketan, 20 μM, $n = 38$, $N = 4$), a 5-HT_{2A} antagonist, and SB269970 (+SB, 20 μM, $n = 46$, $N = 4$), a 5-HT_7 antagonist, were used. 5-HT significantly potentiated the capsaicin-induced $[Ca^{2+}]_i$ increases but it was suppressed by the simultaneous application of a 5-HT_{2A} or 5-HT_7 antagonist. ** $P < 0.01$; NS, not significant. For the experimental protocol, see Results.

between the inflammation and control sides. These results suggest that the increase of the biosynthesis of at least 5-HT_{2A} and 5-HT_7 receptor subtypes may lead to the potentiation of TRPV1 function induced by 5-HT.

Discussion

In the present experiment, 5-HT had a sensitizing action on TRPV1 functions in sensory neurons, since it was

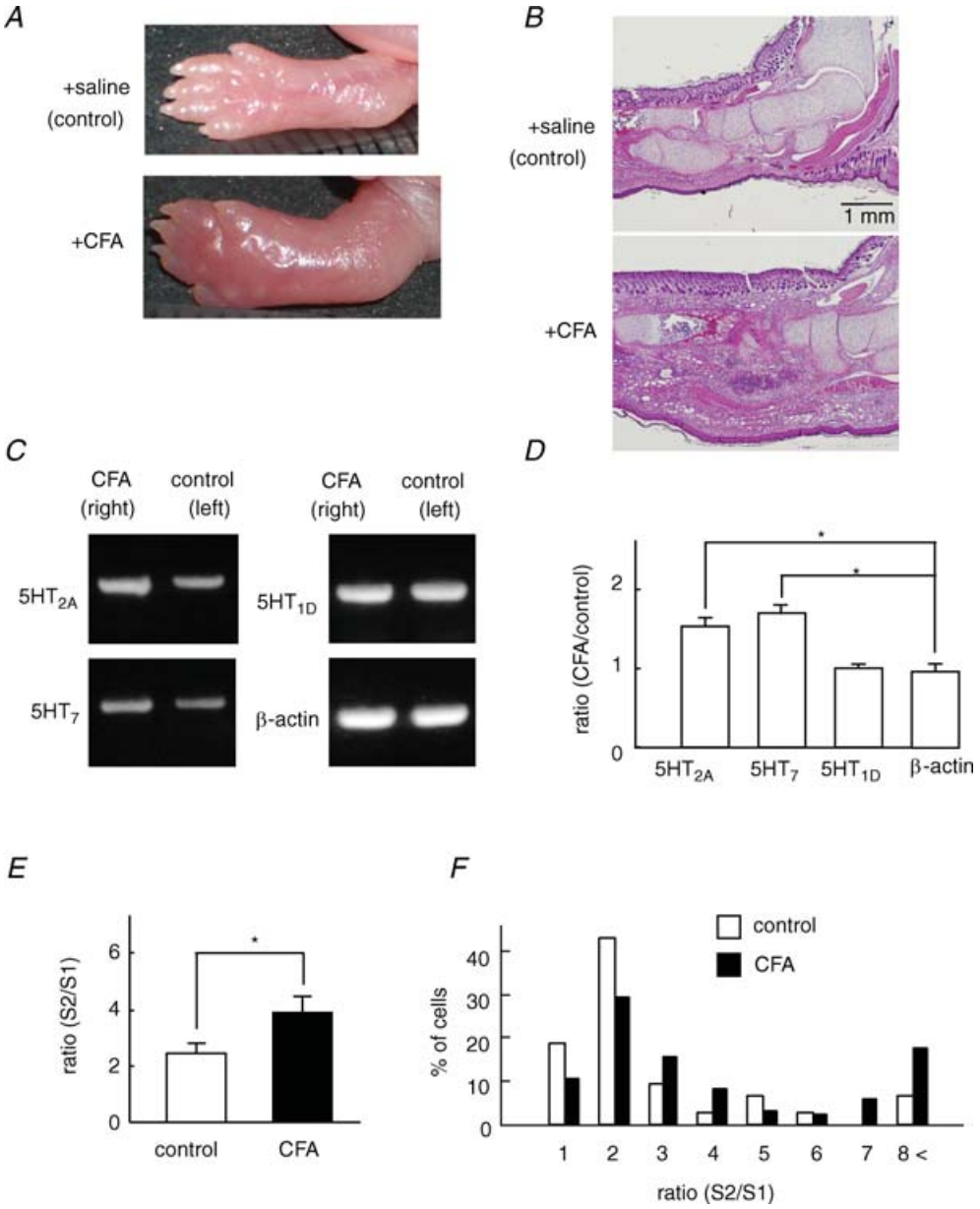

Figure 6. Inflammation increased 5-HT receptor expression and the potentiating effect of 5-HT on $[Ca^{2+}]_i$ responses to capsaicin

A and *B*, complete Freund's adjuvant (CFA) and saline were injected into the right and left hemilateral hind paw of the neonatal rat, respectively. Macroscopic (*A*) and histological (*B*) images 4 days after injection of CFA at the CFA-injected site (+CFA) and saline-injected site (control). *C*, the expression levels of 5-HT_{2A} and 5-HT_7 receptors were more pronounced in DRG isolated from the CFA side than the control side. No significant changes occurred in 5-HT_{1D} and β-actin expression in DRG from both sides. *D*, the ratio of the optical density of PCR products of 5-HT_{2A}, 5-HT_7, 5-HT_{1D} and β-actin from the CFA side to that from the control side ($N = 5$). * $P < 0.05$. *E*, the summarized potentiation rate (S2/S1) estimated by the ratio of the capsaicin (30 nM)-induced $[Ca^{2+}]_i$ increase during the application of 5-HT (S2) to that before 5-HT application (S1). CFA, $n = 114$; control, $n = 69$; $N = 5$. * $P < 0.05$. *F*, histogram of the percentage of cells–S2/S1 ratio relation in response to capsaicin in DRG neurons isolated from the CFA side and control side.

capable of potentiating $[Ca^{2+}]_i$ increases in response to capsaicin, protons and noxious heat. Membrane depolarization and the inward current induced by capsaicin were also increased by 5-HT. Pharmacological and molecular analyses suggested that at least 5-HT_{2A} and 5-HT_7 receptors were involved in 5-HT-induced TRPV1 sensitization through PKC and PKA pathways. Under inflammatory conditions, the expression levels of these 5-HT receptor subtypes increased, and the potentiating effect of 5-HT was augmented, suggesting that sensitization of TRPV1 by 5-HT occurs more markedly under inflammatory conditions. The sensitizing effects of 5-HT on TRPV1 functions may be partly involved in the 5-HT-induced hyperalgesic responses *in vivo*.

In this study, it was clarified that the $[Ca^{2+}]_i$ increase induced by capsaicin was increased by 5-HT in more than 70% of capsaicin-sensitive cells regardless of IB4 staining, though nociceptors having different responsiveness can be cytochemically subdivided by IB4 staining (Dirajlal *et al.* 2003; Liu *et al.* 2004; Breese *et al.* 2005). Similarly, there was no relation between IB4 staining and the potentiating effects of 5-HT on proton- and heat-induced $[Ca^{2+}]_i$ responses. Therefore, functional differences reflected by IB4 staining were not associated with the TRPV1 potentiating effects of 5-HT in neonatal rat DRG neurons.

In addition to the augmentation of $[Ca^{2+}]_i$ responses to capsaicin, membrane depolarization and current responses to capsaicin were also increased by 5-HT. Under current-clamp conditions, capsaicin-induced depolarization was enhanced by 5-HT. In some cells, capsaicin produced a prominent spike discharge superimposed on the increased sustained depolarization in the presence of 5-HT. Moreover, 5-HT augmented proton-induced inward currents. In many DRG neurons, protons evoked inward currents with different kinetics composed of transient and sustained currents. It is reported that a component of the transient inward current evoked by protons is mediated by the activation of acid-sensing ion channels (ASIC), because of being blocked by amiloride (Dirajlal *et al.* 2003; Liu *et al.* 2004). In contrast, the sustained component is mediated predominantly by TRPV1 (Liu *et al.* 2004). In the present results, a sustained component of proton-induced inward current was augmented by 5-HT. This may provide further evidence that 5-HT potentiates TRPV1-mediated responses. Different amino acid residues are responsible for the recognition of capsaicin and protons (Jordt *et al.* 2000). Therefore, it is likely that 5-HT increases TRPV1 function regardless of activating factors. These results suggest that 5-HT may participate in the inflammatory hyperalgesia elicited by extracellular acid and heat during inflammation. Under patch-clamp conditions, the percentage of cells showing potentiating effects by 5-HT was smaller (30–40%) than those in $[Ca^{2+}]_i$ imaging experiments (~70%). It may be due to washout of some cellular components related to 5-HT-induced potentiation of TRPV1 function under patch-clamp conditions.

TRPV1 functions are reported to be potentiated through the PLC/PKC (Cesare *et al.* 1999; Premkumar & Ahern, 2000) and cAMP/PKA pathways (Lopshire & Nicol, 1998; Gu *et al.* 2003; Moriyama *et al.* 2003), respectively. Phosphorylation of TRPV1 by these kinases is related to the TRPV1 sensitization (Numazaki *et al.* 2002; Wang *et al.* 2004; Ferreira *et al.* 2005), and putative phosphorylation sites have been demonstrated (Numazaki *et al.* 2002; Bhave *et al.* 2002; Mohapatra & Nau, 2003). A PKC inhibitor (BIM) or PKA inhibitor (H89) suppressed the TRPV1 sensitization induced by 5-HT, indicating that both kinase pathways are involved in the 5-HT-induced TRPV1 potentiation in rat DRG neurons. However, there was some diversity in the extent of the inhibitory actions of BIM and H89. This may be due to the differences of dominant signalling pathways in neurons or differences in the expression levels of 5-HT receptors coupled to these intracellular signallings. Alternatively, PKC-independent mechanisms may be involved in the 5-HT-induced TRPV1 potentiation, because BK and NGF stimulate PLC and promote hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), relieving TRPV1 from PIP_2-mediated inhibition (Chuang *et al.* 2001). It has been also reported that phosphatidylinositol-3-kinase and calcium–calmodulin-dependent protein kinase II are important in mediating the sensitization of TRPV1 (Bonnington & McNaughton, 2003). The BIM treatment produced not only suppression of the potentiation effect of 5-HT but also reduced the capsaicin-induced $[Ca^{2+}]_i$ increase. PKCγ, an important isoform in the increased pain sensitivity sensitive to Ca^{2+} (Martin *et al.* 2001), can be activated by Ca^{2+} influx through highly Ca^{2+}-permeable TRPV1 channels. It has also been suggested that background PKC activity is sufficient to phosphorylate TRPV1 (Vellani *et al.* 2001) and BIM attenuates the capsaicin-evoked current in rat DRG neurons due to inhibition of basal phosphorylation of TRPV1 (Zhou *et al.* 2001).

RT-PCR analysis demonstrated that 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_3, 5-HT_4, 5-HT_{5B} and 5-HT_7 receptor subtypes were expressed in neonatal rat DRG neurons. These results are essentially consistent with the data reported for adult DRG neurons (Wu *et al.* 2001). The absence of 5-HT_{1A} mRNA is demonstrated in rat lumbar DRG (Chen *et al.* 1998). Pierce *et al.* 1996) reported that 5-HT_{2C}, but not 5-HT_{1F}, 5-HT_4 or 5-HT_{5B}, are present in adult rat DRG. The reasons for these differences are not clear, but they might be due to the age of the animals or sites of DRG tested. The present results were consistent with previous *in situ* hybridization and immunohistochemical data showing that 5-HT_{1B} (Doucet *et al.* 1996), 5-HT_{2A} (Tokunaga *et al.* 1998), 5-HT_3 (Tecott *et al.* 1993; Kia *et al.*

1995) and 5-HT_7 (Meuser *et al.* 2002; Doly *et al.* 2005) are present in rat DRG.

It has been reported that various 5-HT receptor subtypes are related to 5-HT-induced hyperalgesia, as mentioned in the Introduction. Ionotropic 5-HT_3 receptors, which when activated promote $[Ca^{2+}]_i$ increase, are suggested to be responsible for 5-HT-induced pain and hyperalgesia (Eschalier *et al.* 1989; Giordano & Rogers, 1989). In the current study, although a few neurons showed a $[Ca^{2+}]_i$ increase in response to 5-HT, there was no relation between the 5-HT-induced $[Ca^{2+}]_i$ increase and the potentiating action on TRPV1. Therefore, it is unlikely that the potentiating effects of 5-HT on TRPV1 are mediated by ionotropic 5-HT_3 receptors. The 5-HT_{2A} receptor is coupled to the PLC/PKC cascade through $G_{q/11}$ protein and the 5-HT_7 receptor to the AC/PKA cascade through G_s protein (Hoyer *et al.* 2002; Alexander *et al.* 2006). In the present results, the sensitization action of 5-HT was mimicked by the 5-HT_{2A} agonist DOI and the 5-$HT_{1A/7}$ agonist 8OH-DPAT. Furthermore, 5-HT-induced potentiation of the $[Ca^{2+}]_i$ response to capsaicin was significantly suppressed by antagonists for 5-HT_{2A} and 5-HT_7. 8-OH-DPAT has a high affinity for both 5-HT_{1A} and 5-HT_7 receptors, and 5-HT_{1A} and 5-HT_7 receptors have been shown to have similar pharmacological profiles (Shen *et al.* 1993). In the present study, since RT-PCR analysis revealed the absence of 5-HT_{1A} receptor mRNA, it is likely that the potentiating effect of 8OH-DPAT on capsaicin-induced $[Ca^{2+}]_i$ increases was mediated by 5-HT_7 receptors. In the rat, production of c-*fos* in the spinal dorsal horn is increased when a 5-HT_7 receptor agonist is administered (Meuser *et al.* 2002). The present data suggest that 5-HT_7 receptors in part play a role in hyperalgesia induced by 5-HT. It has been reported that TRPV1 function in mouse colon sensory neurons is enhanced by 5-HT through 5-HT_2 and 5-HT_4 receptors (Sugiura *et al.* 2004). In the rat, pain induced by noxious fever, intraperitoneal injection of acetic acid and inflammation of the foot is mediated by 5-HT_4 (Espejo & Gill, 1998). Moreover, the present RT-PCR analysis demonstrated the presence of several 5-HT receptors other than 5-HT_{2A} and 5-HT_7. Therefore, it leaves open the possibility of the involvement of other 5-HT receptor subtypes in TRPV1 sensitization in rat DRG.

By the administration of CFA to the hind paw, remarkable inflammatory responses occurred. Significant increases of the expression levels of 5-HT_{2A} and 5-HT_7 receptor mRNAs, but not that of 5-HT_{1D}, were observed in DRG of the ipsilateral CFA-administered side compared to the contralateral control side. The present results are consistent with a previous report that 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_3, 5-HT_4 and 5-HT_7 receptor mRNA expression levels are increased by CFA inflammation in adult rat DRG (Wu *et al.* 2001). Neurotrophic factors such as NGF (Woolf, 1996) and BDNF (brain-derived neurotrophic factor; Mannion *et al.* 1999) may contribute to the changes in expression of various proteins under inflammation. In addition, we found that TRPV1 augmentation by 5-HT was more marked in DRG neurons of the inflammation side. These results suggest that the hypersensitivity of nociceptors induced by 5-HT may be mediated by increased expression levels of 5-HT receptor subtypes, at least 5-HT_{2A} and 5-HT_7, under inflammatory conditions. The present findings provide information about potential peripheral mechanisms that contribute to inflammatory hyperalgesia mediated by 5-HT. Additional studies are needed to determine whether inhibition of TRPV1 functions or their modulation through 5-HT and/or other signalling pathways may be useful therapeutic targets in the treatment of inflammatory pain syndromes.

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