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# Involvement of Transient Receptor Potential Vanilloid Subtype 1 in Analgesic Action of Methylsalicylate[S]

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## ABSTRACT

Methylsalicylate (MS) is a naturally occurring compound that is used as a major active ingredient of balms and liniments supplied as topical analgesics. Despite the common use of MS as a pain reliever, the underlying molecular mechanism is not fully understood. Here we characterize the action of MS on transient receptor potential V1 (TRPV1). In human embryonic kidney 293 cells expressing human TRPV1 (hTRPV1), MS evoked increases of $[Ca^{2+}]_i$, which declined regardless of its continuous presence, indicative of marked desensitization. TRPV1 antagonists dose-dependently suppressed the MS-induced $[Ca^{2+}]_i$ increase. MS simultaneously elicited an inward current and increase of $[Ca^{2+}]_i$ in the voltage-clamped cells, suggesting that MS promoted $Ca^{2+}$ influx through the activation of TRPV1 channels. MS reversibly inhibited hTRPV1 activation by polymodal stimuli such as capsaicin, protons, heat, anandamide, and 2-aminoethoxydiphenyl borate. Because both the stimulatory and inhibitory actions of MS were exhibited in capsaicin- and allicin-insensitive mutant channels, MS-induced hTRPV1 activation was mediated by distinct channel regions from capsaicin and allicin. In cultured rat sensory neurons, MS elicited a $[Ca^{2+}]_i$ increase in cells responding to capsaicin. MS significantly suppressed nocifensive behavior induced by intraplantar capsaicin in rats. The present data indicate that MS has both stimulatory and inhibitory actions on TRPV1 channels and suggest that the latter action may partly underlie the analgesic effects of MS independent of inhibition of cyclooxygenases in vivo.

Transient receptor potential vanilloid subtype 1 (TRPV1) is a nonselective cation channel with high permeability to $Ca^{2+}$ in sensory neurons and functions as a molecular integrator of pain perceptions (Caterina et al., 1997). TRPV1 is activated by polymodal stimuli such as capsaicin, protons, heat, and anandamide, an endogenous vanilloid (Starowicz et al., 2007). Because mice genetically lacking TRPV1 exhibit impaired nociception (Caterina et al., 2000; Davis et al., 2000), and several TRPV1 antagonists show antinociceptive activity in vivo (Pomonis et al., 2003; Walker et al., 2003; Gavva et al., 2005), TRPV1 is considered to have an important role in the signal transduction pathways of the nociceptive system.

Many naturally occurring pungent compounds have been reported to be activators of TRPV1 (Calixto et al., 2005). Agonists of TRPV1 can induce a long-lasting analgesic effect due to desensitization after initial activation (Koplas et al., 1997; Bhave et al., 2002). Methylsalicylate (MS), known as salicylic acid methyl ester or oil of wintergreen, is a plant-derived natural product extracted from some species of the shrub genus *Gaultheria* in the closely related family Ericaceae. MS is widely available over the counter and is a common ingredient for liniments, ointments, and essential oils used as topical ointments or medicated oils for the relief of musculoskeletal aches and pains. Salicylate derivatives such as salicylic acid and acetylsalicylic acid (aspirin), are recognized as nonsteroidal anti-inflammatory drugs (NSAIDs) used in the treatment of inflammation and pain in a wide variety of disorders (Cashman and McAnulty, 1995; Wu, 1998). The best known mechanism of action of NSAIDs is the inhibition of prostaglandin synthesis caused by their action on cyclooxygenases (COXs) (Vane and Botting, 2003). However, because it has been shown that NSAIDs also act on targets other than COXs to counteract pain

**ABBREVIATIONS:** TRPV1, transient receptor potential vanilloid 1; 2APB, 2-aminoethoxydiphenylborate; COX, cyclooxygenase; DRG, dorsal root ganglion; FRET, fluorescence resonance-energy transfer; GFP, green fluorescent protein; HEK, human embryonic kidney; IRTX, iodoresiniferatoxin; PIP$_2$, phosphatidyl 4,5-bisphosphate; MS, methylsalicylate; NSAID, nonsteroidal anti-inflammatory drug; TRPA1, transient receptor potential A1; BCTC, *N*-(4-*t*-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carboxamide.

(Voilley, 2004), their analgesic effects are not necessarily the consequence of their anti-COX action. Despite the well known analgesic action of MS, the underlying molecular mechanisms independent of COX inhibition have not been demonstrated.

It has been reported that MS activates heterologously expressed TRPA1 (Bandell et al., 2004), a member of the TRP channel family expressed by nociceptors and its potential role as a sensor of noxious cold (Story et al., 2003; Bandell et al., 2004). We reported recently that mustard oil or allyl isothiocyanate, which is known as a TRPA1 agonist, had an agonistic action on TRPV1 (Ohta et al., 2007). We suggested that TRPV1 was partly related to neurogenic inflammation induced by mustard oil in vivo. For this analogy, we hypothesized that TRPV1 might play a role as a molecular target for MS. Thus, we investigated the effects of MS on TRPV1 using a heterologous expression system and sensory neurons.

Here we show that MS has both stimulatory and inhibitory actions on heterologously expressed human and porcine TRPV1. Because MS reversibly inhibited TRPV1 activation by polymodal stimuli and capsaicin-induced nocifensive behavior, we suggest that the inhibitory action of MS on TRPV1 is one of the underlying mechanisms for its analgesic effects in vivo.

## Materials and Methods

**Cell Preparation, DNA Cloning, and Transfection.** All protocols for experiments on animals were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University. Dorsal root ganglion (DRG) neurons were obtained from rats as described previously (Ohta et al., 2006). In brief, Wistar rats (1–4 days old) were euthanized with $CO_2$ inhalation. The lumbar DRG were removed and enzymatically dissociated with collagenase (1 mg/ml, type II; Worthington Biochemicals, Freehold, NJ) and DNase (1 mg/ml; Roche, Indianapolis, IN) for 30 min at 37°C. Then, ganglia were further digested with trypsin (10 mg/ml; Sigma, St. Louis, MO) and DNase (1 mg/ml) for 30 min at 37°C. Isolated cells were plated onto glass coverslips coated with poly(D-lysine) (Sigma) and cultured in M199 (Sigma) supplemented with fetal bovine serum (10%; Sigma), penicillin G (100 U/ml; Meiji-Seika, Singapore), and streptomycin (100 μg/ml; Banyu, Tokyo, Japan) in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. The cells were used within 1 day after preparation.

Human TRPV1 (hTRPV1) was cloned from a human spinal cord cDNA library (Clontech, Mountain View, CA) based on a published sequence (GenBank accession no. NM_080704) as described previously (Ohta et al., 2008). We used the pcDNA-DEST53 Gateway vector, which contains the "cycle 3 mutant" of the green fluorescent protein (GFP) gene as the N terminus in vector sequences. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cells were transfected with the expression vector using a transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA) and used 24 to 48 h after transfection. GFP-tagged porcine TRPV1 (pTRPV1)-expressing HEK 293 cells were prepared as described previously (Ohta et al., 2007). The TRPV1-expressing cells were detected by GFP fluorescence with appropriate filters. Single amino acid mutations of hTRPV1 were made using a PrimeSTAR Mutagenesis Basal Kit (Takara, Kyoto, Japan) with the following sets of primers: for Y511A: forward, 5′-GACAGCGCCAGTGAGATGCTTT-TCTTT-3′, and reverse, 5′-CTCACTGGCGCTGTCCACAAACAGGGT-3′; for S512Y: forward, 5′-AGCTACTATGAGATGCTTTTCTTTCTG-3′, and reverse, 5′-CATCTCATAGTAGCTGTTCACAAACAG-3′; and for C157A: forward, 5′-AAGACCGCTCTGCTGAAAGCCATGCTC-3′, and reverse, 5′-CAGCAGAGCGGTCTTCCCTGTCTCAGG-3′. All clones

and mutants used were verified by sequencing using an automated sequencer (AB1 PRISM 3100; Applied Biosystems, Foster City, CA). Normal human epidermal keratinocytes of neonatal foreskins were purchased from Kurabo (Osaka, Japan). The cells were cultured in serum-free keratinocyte growth medium consisting of Humedia-KB2 (Kurabo) supplemented with bovine pituitary extract [0.4% (v/v)], human recombinant epidermal growth factor (0.1 ng/ml), insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 ng/ml). The medium was replaced every 2 days.

**$Ca^{2+}$ Imaging.** The intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) in individual cells were measured with a fluorescent $Ca^{2+}$ indicator, fura-2, by dual excitation using a fluorescent imaging system controlling illumination and acquisition (Aqua Cosmos; Hamamatsu Photonics, Shizuoka, Japan) as described previously (Ohta et al., 2006). To load fura-2, cells were incubated for 30 min at 37°C with 10 μM fura-2 acetoxymethyl ester in normal external solution consisting of 134 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 10 mM HEPES, pH 7.4, with NaOH. For $Ca^{2+}$-free external solution, $Ca^{2+}$ was omitted, and EGTA (0.5 mM) was added. A coverslip with dye-loaded cells was placed in an experimental chamber (~0.5 ml) mounted on the stage of an inverted microscope (Olympus IX71; Olympus, Tokyo, Japan) equipped with an image acquisition and analysis system. To measure $[Ca^{2+}]_i$, cells were illuminated every 2.5 s with light at 340 and 380 nm, and the respective fluorescent signals of 500 nm were detected. The fluorescence emitted was projected to a charge-coupled device camera (ORCA-ER; Hamamatsu Photonics) and the ratios of fluorescent signals ($F_{340}/F_{380}$) for $[Ca^{2+}]_i$ were stored on the hard disc of a personal computer (LF-D521; Panasonic, Tokyo, Japan). In some experiments, the change in $[Ca^{2+}]_i$ was measured using *cameleon* (Miyawaki et al., 1997), which is a fluorescence resonance-energy transfer (FRET)-based $Ca^{2+}$ sensor. HeLa cells were transfected with *cameleon* delivered by baculovirus-based transduction (Premo Cameleon; Invitrogen, Carlsbad, CA) and hTRPV1 cDNA (hTRPV1-pGATE; enhanced green fluorescent protein-removed expression vector). For measurement of ratiometric FRET signals, cells were illuminated with light at 450 nm ($F_{450}$), yellow fluorescent protein emission ($F_{480}$), and cyan fluorescent protein emission ($F_{535}$) were detected. Cells were superfused continuously with the external solution at a flow rate of ~1.5 ml/min through a Y-tube pipette placed close to the cells. All experiments were carried out at room temperature (22–24°C).

**Whole-Cell Current Recording.** Membrane currents were recorded from GFP-positive HEK cells using the conventional whole-cell configuration of the patch-clamp technique with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA). The resistance of patch electrodes ranged from 4 to 5 MΩ. The currents were filtered at 1 kHz and sampled at 5 kHz using an A/D converter (Digidata 1322A; Molecular Devices) in conjunction with a personal computer. During the experiments and analysis, pClamp 6 software (Molecular Devices) was used. The standard pipette solution contained 120 mM CsCl, 20 mM tetraethylammonium chloride, 1.2 mM $MgCl_2$, 2 mM $ATPNa_2$, 0.2 mM $GTPNa_3$, 10 mM HEPES, and 10 mM EGTA, pH 7.2, with CsOH. For simultaneous measurement of $[Ca^{2+}]_i$ with the current responses, the pipette solution contained the following: 120 mM CsCl, 20 mM tetraethylammonium chloride, 1.2 mM $MgCl_2$, 2 mM ATP-$Na_2$, 0.2 mM GTP-$Na_3$, 10 mM HEPES, and 0.1 fura-2, pH 7.2, with CsOH.

**Behavioral Test.** Male Wistar rats (8 weeks old) were acclimated for 30 min in individual plastic chambers before behavioral experiments in a temperature- and humidity-controlled room. To observe TRPV1-induced spontaneous pain behavior, 30 μg/ml capsaicin was injected into a hind paw of each rat in a volume of 20 μl (0.5 μg). The capsaicin-induced nocifensive behavior (paw licking, biting, and flinching) was quantified for 5 min. MS (0.5 mg) was injected into the same hind paw 5 min before injection of capsaicin. As a control, the same amount of saline was injected instead of MS.

**Chemicals.** Capsaicin from Sigma was dissolved in ethanol at a high concentration as a stock solution (10 mM). (5Z,8Z,11Z,14Z)-*N*-

(2-hydroxyethyl) icosa-5,8,11,14-tetraenamide (anandamide), capsazepine, 2-aminoethoxydiphenylborate (2APB), iodoresiniferatoxin (IRTX), ionomycin, ruthenium red, and wortmannin were from Sigma, and $N$-(4-$t$-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2$H$)-carboxamide (BCTC) was from BIOMOL Research Laboratories (Plymouth Meeting, PA), and acrolein was from AccuStandard (New Haven, CT) and dissolved in dimethyl sulfoxide to make a stock solution (0.1 M). Allicin from LKT Laboratories (St. Paul, MN), allyl isothiocyanate, and cinnamaldehyde from Nakarai (Tokyo, Japan), and MS from Sigma were dissolved in the external solution and sonicated before use. These drugs did not change the pH or osmolality of the external solution. All other drugs used were of analytical grade.

**Data Analysis.** The data are presented as the mean ± S.E.M. ($n$ = number of observations). Comparisons were made by the paired or unpaired Student's $t$ test and differences with a $P$-value of less than 0.05 were considered significant. Pairwise associations were examined by Pearson's linear regression. Values of the 50% effective ($EC_{50}$) and inhibitory ($IC_{50}$) concentrations were determined using Origin software (OriginLab Corp, Northampton, MA). The present data were obtained from at least three different transfections per experiment.

## Results

**Methylsalicylate Increases $[Ca^{2+}]_i$ in Human TRPV1-Expressing HEK 293 Cells.** Because the TRPV1 channel has high $Ca^{2+}$ permeability (Caterina and Julius, 2001), we examined whether MS could stimulate TRPV1 channels using $Ca^{2+}$ imaging with fura-2 (Fig. 1). Fura-2-loaded hTRPV1-expressing HEK 293 cells were first stimulated with capsaicin (30 nM) and subsequently with MS (3 mM). Representative time-lapse $[Ca^{2+}]_i$ imaging showed that MS elicited a $[Ca^{2+}]_i$ increase in cells responding to capsaicin (Fig. 1A). The time-to-peak of the $[Ca^{2+}]_i$ increase induced by MS was slower (29.5 ± 3.1 s, $n$ = 85, $p < 0.01$) than that induced by capsaicin (19.2 ± 1.1 s). A similar magnitude of $[Ca^{2+}]_i$ was elicited by the second application of capsaicin or MS (Fig. 1B). As shown in Fig. 1C, there was a significantly positive relation between the amplitude of the $[Ca^{2+}]_i$ increase induced by capsaicin and that induced by MS in each

hTRPV1-expressing HEK 293 cell ($r = 0.68$, $p < 0.01$). The average change in $[Ca^{2+}]_i$ increase induced by MS (3 mM) was approximately half of that induced by capsaicin (30 nM) (Fig. 1D). MS evoked $[Ca^{2+}]_i$ increases in hTRPV1-expressing HEK 293 cells in a concentration-dependent manner (Fig. 2, A and B). When cells were exposed to high concentration of MS (30 mM), not only an increment of $[Ca^{2+}]_i$ but also irreversible cell swelling occurred regardless of whether there was TRPV1 expression or not (data not shown).

In the present experiment, we mainly analyzed the hTRPV1 function using a recombinant channel with a GFP fusion protein (GFP-hTRPV1). To negate the possibility that MS elicited the $[Ca^{2+}]_i$ increase in heterologously hTRPV1-expressing cells as a result of some physiochemical interaction with GFP protein or with the fluorescent $Ca^{2+}$ indicator fura-2, we constructed a GFP-free expression plasmid and transfected it into HeLa cells expressing a FRET-based $Ca^{2+}$ sensor, *cameleon* (Supplemental Fig. 1). MS and capsaicin changed FRET signals and increased the ratio, which reflected the $[Ca^{2+}]_i$ increase in these cells. These data excluded the possibility that GFP fusion affected the nature of hTRPV1, methods for $[Ca^{2+}]_i$ measurement, or cellular dependence used for transfection in the $[Ca^{2+}]_i$ responses to MS.

Because MS was reported to be an agonist for TRPA1 (Bandell et al., 2004; Calixto et al., 2005), we examined whether recombinant hTRPV1 had a relation to TRPA1 function by investigating several chemicals known as TRPA1 agonists (Bautista et al., 2006). Cinnamaldehyde (1 mM) and acrolein (0.05 mM) did not increase $[Ca^{2+}]_i$ (Supplemental Fig. 2). However, another TRPA1 agonist, allyl isothiocyanate (mustard oil), produced an $[Ca^{2+}]_i$ increase. We found recently that this compound had an agonistic action on TRPV1 (Ohta et al., 2007). These cells confirmed the responsiveness to MS (3 mM) and capsaicin (30 nM).

It is known that the capsaicin-evoked $[Ca^{2+}]_i$ increase results from an influx of divalent cations directly through TRPV1 channels (Caterina and Julius, 2001). In addition,
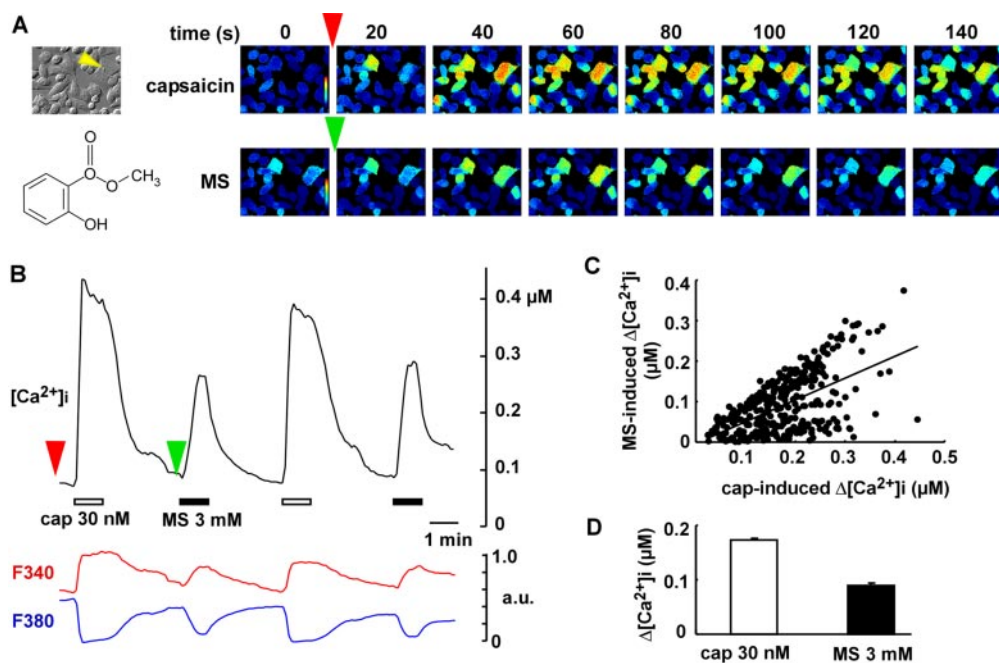
**Fig. 1.** Methylsalicylate increases $[Ca^{2+}]_i$ in HEK 293 cells heterologously expressing human TRPV1. A, left, an image under transmitted light, and the chemical structure of MS. Right, pseudocolor time-lapse images from microscope-based ratiometric analysis of $[Ca^{2+}]_i$ corresponding to the time points described in B. B, representative $[Ca^{2+}]_i$ increases induced by repetitive applications of capsaicin (cap, 30 nM) and MS (3 mM) in the same cell (shown by the yellow arrowhead in A). Top, $[Ca^{2+}]_i$ and the lower fluorescent signals of fura-2 exiting at 340 and 380 nm; a.u., arbitrary unit. C, relationship between the amplitude of MS- and capsaicin-induced $[Ca^{2+}]_i$ increases in individual cells ($n$ = 253). D, averaged peak amplitude of $[Ca^{2+}]_i$ increases induced by capsaicin and MS. Symbols with vertical lines show mean ± S.E.M.

there are some reports that $Ca^{2+}$ release from internal stores is involved in the $[Ca^{2+}]_i$ increase after TRPV1 activation (Marshall et al., 2003; Karai et al., 2004). To elucidate whether MS-induced $[Ca^{2+}]_i$ increases were linked to the internal $Ca^{2+}$ stores, the effects of $Ca^{2+}$ removal on $[Ca^{2+}]_i$ responses to MS were examined. As shown in Fig. 2C, no $[Ca^{2+}]_i$ increase occurred with the application of MS or capsaicin in the absence of external $Ca^{2+}$ ($[Ca^{2+}]_o$). These results suggested that $[Ca^{2+}]_i$ responses to MS were related to $Ca^{2+}$ influx across the plasma membrane rather than $Ca^{2+}$ mobilization of internal stores.
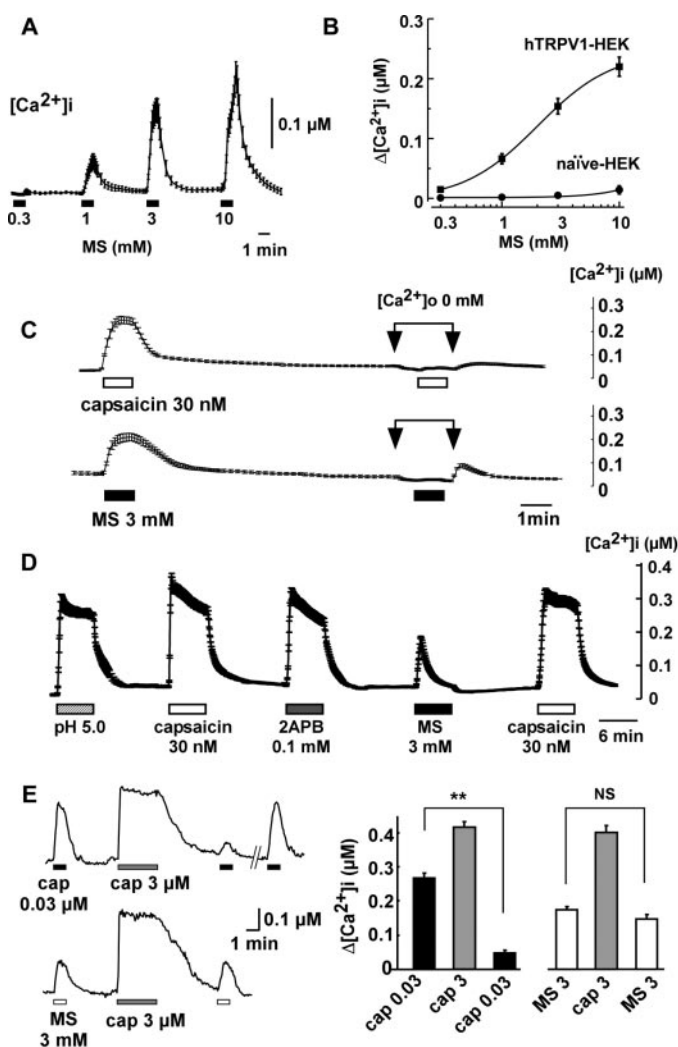


**Fig. 2.** Characterization of methylsalicylate-induced $[Ca^{2+}]_i$ increases. A, averaged changes in $[Ca^{2+}]_i$ in response to MS at different concentrations for 1 min ($n = 43$). B, concentration-response relationship for MS in hTRPV1-expressing HEK 293 cells (hTRPV1-HEK, $n = 68$) and nontransfected cells (naive-HEK, $n = 61$). C, effects of external $Ca^{2+}$ removal of MS-induced $[Ca^{2+}]_i$ increase. External $Ca^{2+}$ was removed by changing normal external solution to $Ca^{2+}$-free solution 0.5 min before stimulation with capsaicin (top, $n = 40$) or MS (bottom, $n = 45$). D, averaged $[Ca^{2+}]_i$ responses to various TRPV1 agonists, pH 5.0 (capsaicin 30 nM, 2APB 0.1 mM), and MS (3 mM) for 6 min in the same cells ($n = 50$). E, desensitization of $[Ca^{2+}]_i$ responses to capsaicin but not to MS after stimulation with a supramaximal concentration of capsaicin. Left, top, typical $[Ca^{2+}]_i$ responses induced by capsaicin (cap; 0.03 $\mu$M) for 1 min before and after 3 $\mu$M capsaicin exposure for 3 min. Bottom, those induced by MS (3 mM). Right, summarized effects of capsaicin (0.03 $\mu$M) and MS (3 mM) before and after the application of capsaicin (3 $\mu$M). ∗∗, $p < 0.01$ versus the response before application of 3 $\mu$M capsaicin. NS, not significant. Symbols with vertical lines show mean ± S.E.M.

To further characterize the MS-induced $[Ca^{2+}]_i$ increase in hTRPV1-expressing cells, we compared $[Ca^{2+}]_i$ responses of three different TRPV1 stimuli; low pH of 5.0, capsaicin (30 nM), and 2-aminoethoxydiphenyl borate (2APB), which is a common agonist for TRPV1, TRPV2, and TRPV3 channels (Hu et al., 2004). These stimuli were applied for 6 min to the same cells expressing hTRPV1 (Fig. 2D). Except for MS, $[Ca^{2+}]_i$ was sustained during the presence of each stimulation. The MS-induced $[Ca^{2+}]_i$ increase markedly declined despite its continuous presence. This was not due to an irreversible loss of cellular responsiveness, because $[Ca^{2+}]_i$ responses to capsaicin of almost the same amplitude occurred after washout of MS. The percentages of the $[Ca^{2+}]_i$ levels at 6 min after their application to each maximal response were 70.3 ± 1.3% for capsaicin and 8.2 ± 2.5% for MS ($n = 42$), suggesting that MS produced short-term desensitization of TRPV1.

To examine whether cross-desensitization occurred in $[Ca^{2+}]_i$ responses to capsaicin and MS, capsaicin (0.03 $\mu$M) or MS (3 mM) was applied to cells after stimulation with a supramaximal concentration of capsaicin (3 $\mu$M). As shown in Fig. 2E, capsaicin (0.03 $\mu$M)-induced $[Ca^{2+}]_i$ increases were significantly reduced after the application of the high concentration of capsaicin, indicative of the desensitization of capsaicin-induced responses. In contrast, the $[Ca^{2+}]_i$ response to MS (3 mM) was almost unchanged even after 3 $\mu$M capsaicin stimulation.

It has been reported that phosphatidyl 4,5-bisphosphate (PIP$_2$) negatively regulates TRPV1 activity (Chuang et al., 2001; Liu et al., 2005). High concentrations of wortmannin cause depletion of PIP$_2$ by inhibiting resynthesis of the phospholipids through phosphatidylinositol 4-kinase (Suh and Hille, 2002; Liu et al., 2005), resulting in an increase of capsaicin-induced $[Ca^{2+}]_i$ responses (Vetter et al., 2008). Thus, we examined whether MS-induced TRPV1 activation was subject to PIP$_2$ regulation. Cells were pretreated with a high concentration of wortmannin (100 $\mu$M) for 20 min. The pretreatment with wortmannin, however, did not significantly affect $[Ca^{2+}]_i$ responses to MS ($\Delta[Ca^{2+}]_i$; control, 102.0 ± 7.1 nM, and treated cells, 104.6 ± 6.0 nM, $n = 71$) or those to capsaicin ($\Delta[Ca^{2+}]_i$; control, 146.0 ± 6.3 nM, and treated cells, 155.9 ± 5.9 nM, $n = 84$).

**Pharmacological Characterization of $[Ca^{2+}]_i$ Responses to Methylsalicylate.** We next examined the effects of TRPV1 antagonists for the ability to inhibit the MS-induced $[Ca^{2+}]_i$ increase. Figure 3A shows averaged $[Ca^{2+}]_i$ responses to capsaicin and MS before, during, and after the application of capsazepine (1 $\mu$M), a competitive TRPV1 antagonist. Capsazepine abolished both capsaicin- and MS-induced $[Ca^{2+}]_i$ increases, which were almost completely restored after washout of capsazepine. The concentration-inhibition curves for four different TRPV1 antagonists, IRTX, BCTC, capsazepine, and ruthenium red, in the $[Ca^{2+}]_i$ response to MS are shown in Fig. 3B. For comparison, the effects of these antagonists on the capsaicin-induced $[Ca^{2+}]_i$ increase was also examined (Fig. 3C). Inhibitory effects of these antagonists were examined by the application of increasing concentrations together with MS (3 mM) or capsaicin (30 nM). All of the antagonists inhibited both MS- and capsaicin-induced responses in a dose-dependent manner. The IC$_{50}$ values of TRPV1 antagonists for responses to capsaicin were 0.11 ± 0.01 nM ($n = 60$) for IRTX, 0.97 ± 0.10 nM

($n = 45$) for BCTC, $7.26 \pm 1.71$ nM ($n = 55$) for capsazepine, and $12.3 \pm 1.3$ nM ($n = 69$) for ruthenium red. $IC_{50}$ values for responses to MS were $0.89 \pm 0.08$ nM ($n = 51$) for IRTX, $3.46 \pm 0.51$ nM ($n = 48$) for BCTC, $5.68 \pm 0.91$ nM ($n = 42$) for capsazepine, and $13.6 \pm 2.6$ nM ($n = 59$) for ruthenium red. These data led to the suggestion that MS evoked a $[Ca^{2+}]_i$ increase through the activation of TRPV1 channels.

In the pharmacological characteristics of TRPV1, there are some species differences: chicken TRPV1 is not sensitive to capsaicin (Jordt and Julius, 2002), and rabbit TRPV1 is less sensitive to capsaicin (Gavva et al., 2004). Therefore, to clarify whether MS stimulated TRPV1 channels other than the human ortholog, the effects of MS on pTRPV1-expressing HEK 293 cells were examined (Supplemental Fig. 3). MS produced a $[Ca^{2+}]_i$ increase in cells responding to capsaicin in pTRPV1-expressing cells with potency similar to that of hTRPV1. Capsazepine reversibly suppressed both capsaicin- and MS-induced $[Ca^{2+}]_i$ increases, indicating that MS stimulated not only the human but also the porcine ortholog of TRPV1 channels.

To determine whether MS had an agonistic action on endogenous hTRPV1, we examined the effects of MS on cultured human keratinocytes, which have been reported to express functional TRPV1 (Denda et al., 2001; Inoue et al., 2002). However, no $[Ca^{2+}]_i$ response occurred with capsaicin in human keratinocytes, even at a high concentration (0.1 mM). Moreover, MS (3 mM) did not increase $[Ca^{2+}]_i$, but camphor (10 mM) and 2APB (1 mM) did (Supplement Fig. 4).

**Simultaneous Measurement of Membrane Current and $[Ca^{2+}]_i$ Responses to Methylsalicylate.** The $[Ca^{2+}]_i$ increase induced by capsaicin is known to be mediated by an influx of divalent cations directly through TRPV1 channels. To obtain direct evidence that the MS-induced $[Ca^{2+}]_i$ increase is mediated by a promotion of $Ca^{2+}$ influx via TRPV1 channels like capsaicin, we simultaneously measured $[Ca^{2+}]_i$



**Fig. 3.** Inhibitory effects of TRPV1 antagonists on MS-induced increases of $[Ca^{2+}]_i$. A, averaged inhibitory effect of capsazepine (1 $\mu$M) on the capsaicin- and MS-induced $[Ca^{2+}]_i$ increases in the same cells ($n = 50$). B and C, concentration-inhibition curves for effects of IRTX, BCTC, capsazepine (capz), and ruthenium red (RR) on the MS (3 mM)- and capsaicin (30 nM)-induced $[Ca^{2+}]_i$ increases. Responses were estimated as a percentage of the amplitude of the $[Ca^{2+}]_i$ increase by MS or capsaicin in the absence of TRPV1 antagonists ($n = 42-69$). Symbols with vertical lines show the mean $\pm$ S.E.M.

and current responses to MS under voltage-clamp conditions. At a holding potential of $-60$ mV, MS (3 mM) evoked an inward current with a concomitant increase of $[Ca^{2+}]_i$. In the same cell, capsaicin (30 nM) produced a larger inward current together with a $[Ca^{2+}]_i$ increase (Fig. 4A). The changes in the $[Ca^{2+}]_i$ increase and inward current were $0.09 \pm 0.02$ $\mu$M and $3.2 \pm 0.4$ pA/pF for MS ($n = 6$), and $0.20 \pm 0.05$ $\mu$M and $9.2 \pm 1.2$ pA/pF for capsaicin, respectively. To characterize MS-activated currents, we analyzed the current-voltage (I-V) relationship using a voltage-ramp protocol ($-80$ to $+100$ mV for 100 ms) applied every 10 s. MS-activated currents were found to be indistinguishable from those of capsaicin. The I-V relation obtained for MS was outwardly rectifying similar to capsaicin and exhibited a reversal potential close to 0 mV ($E_{rev} = +6.9 \pm 1.2$ compared with $+8.0 \pm 1.1$ mV for capsaicin, $n = 6$) as expected for nonselective cation channels. Time-lapse $[Ca^{2+}]_i$ imaging of capsaicin- and MS-induced responses in a voltage-clamped cell are shown in Fig. 4C. These results indicate that MS activated functional ion channels with $Ca^{2+}$ permeability in hTRPV1-expressing HEK 293 cells.

**Inhibitory Action of Methylsalicylate on TRPV1-Mediated Responses to Polymodal Stimuli.** As shown in Fig. 2E, the MS-induced $[Ca^{2+}]_i$ increase in hTRPV1-expressing cells exhibited more acute desensitization than capsaicin. TRPV1 desensitization is considered to contribute to the analgesic effects of TRPV1 agonists (Tominaga and Tominaga, 2005). Because MS is known as a pain reliever, it is conceivable that the desensitization of MS-induced hTRPV1-mediated responses may contribute to its analgesic properties. Thus, we examined whether TRPV1 activation by various TRPV1 agonists affected the period of MS-induced TRPV1 desensitization. Figure 5A shows typical $[Ca^{2+}]_i$ responses to capsaicin before, during, and after washout of MS. The capsaicin-induced $[Ca^{2+}]_i$ increase was almost abolished during the period when the MS response disappeared in its continuous presence. Summarized effects of MS on the responses to various TRPV1 stimuli are depicted in Fig. 5B. Not only the $[Ca^{2+}]_i$ response to capsaicin, but also those to protons, pH 5.0, high temperature (50°C) and 2APB (0.1 mM) were reversibly inhibited by MS. When MS was applied during the sustained $[Ca^{2+}]_i$ increases in the presence of capsaicin or protons, $[Ca^{2+}]_i$ slightly increased just after its application and then decreased gradually (Fig. 5C). For capsaicin-induced responses, the reduced $[Ca^{2+}]_i$ level was partly restored after washout of MS. However, the proton-induced increase of $[Ca^{2+}]_i$ was completely abolished even after washout of MS. The $[Ca^{2+}]_i$ response to protons was partially restored 12 min after washout of MS. The inhibitory action of MS on capsaicin-induced $[Ca^{2+}]_i$ responses was also observed in hTRPV1-expressing HeLa cells via FRET-based $Ca^{2+}$-imaging (Supplement Fig. 1) and in pTRPV1-expressing HEK 293 cells (Supplement Fig. 3A).

Next, we examined the effect of MS on the $[Ca^{2+}]_i$ response to anandamide, an endogenous TRPV1 agonist (Starowicz et al., 2007). Unlike capsaicin, anandamide produced a sustained $[Ca^{2+}]_i$ increase that hardly returned to the original level after its washout (Fig. 6A, inset). The concentration-response relation of anandamide is shown in Fig. 6A, and the $EC_{50}$ value was $1.21 \pm 0.14$ $\mu$M ($n = 45$). For this distinctive response of anandamide, we examined the effect of MS on anandamide-induced $[Ca^{2+}]_i$ responses by comparing anan-
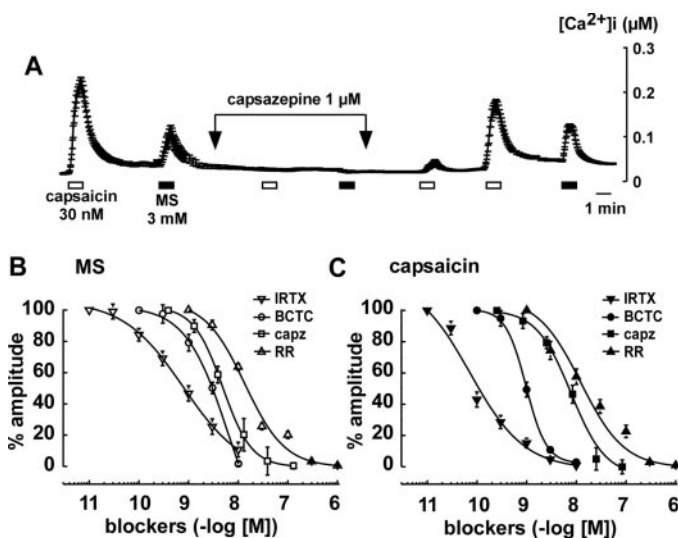
damide effects without (time-matched control) and with 3 mM MS (Fig. 6B). During the period after MS-induced responses disappeared, almost no response was induced by anandamide.

To confirm MS-induced TRPV1 channel activation and desensitization, we measured membrane currents using the whole-cell patch-clamp technique. In hTRPV1-expressing cells, capsaicin and MS evoked both inward and outward currents during voltage ramps (100 ms) from $-80$ to $+100$ mV (Fig. 7A). There is a significant relationship between the amplitude of the current response to capsaicin and MS at $-60$ mV ($r = 0.89$, $p < 0.01$), and the MS-induced inward current was approximately one half of the capsaicin-induced one (Fig. 7D). Similar to $[Ca^{2+}]_i$ responses, current responses to MS decreased during its continuous presence, and the capsaicin-induced current was suppressed significantly (Fig. 7B). MS did not change the reversal potential for capsaicin (Fig. 7C). MS also had an inhibitory effect on the current response to protons, as on the $[Ca^{2+}]_i$ responses (Fig. 8A); that is, proton-induced current was first augmented after the application of MS and then suppressed (Fig. 8C). Again, MS did not change the reversal potentials for proton-induced current (Fig. 8B). These data indicated that MS inhibited hTRPV1 channel activation.

**Segregation of Methylsalicylate Sensitivity to TRPV1 from Capsaicin and Allicin Sensitivity.** Both capsaicin and capsazepine have been suggested to interact with a vanilloid-binding domain composed of transmembrane domains 3 and 4 and their respective cytosolic interfaces (Jordt and Julius, 2002; Gavva et al., 2004). In the preset data, capsazepine effectively suppressed both capsaicin- and MS-induced TRPV1 activation. It has been suggested that tyrosine 511 (Tyr511) and serine 512 (Ser512), located at the transition between the second intracellular loop and the third transmembrane domain, interact with vanilloid ligands at the intracellular face of the membrane (Gavva et al., 2004). Therefore, to investigate whether MS activated hTRPV1 through a vanilloid-dependent mechanism, we studied the effects of MS on single-point mutants of TRPV1 (Y511A and S512Y). These two mutant channels showed nearly no responsiveness to capsaicin (1 $\mu$M) as reported previously (Jordt and Julius, 2002; Sutton et al., 2005). Protons and 2APB evoked $[Ca^{2+}]_i$ increases in these mutants, although the responsiveness in Y511A was smaller than in wild-type channels. We were surprised to find that both vanilloid-insensitive mutant channels displayed MS sensitivity. Moreover, MS-induced desensitization was unchanged in the mutant channels (Fig. 9, C and D).

Quite recently, Salazar et al. (2008) reported that allicin, a pungent extract from onion and garlic, stimulated TRPV1 channels via acting on a single N-terminal cysteine (Cys157). Thus, to elucidate that the action of MS is related to this amino acid, we examined the effects of MS on mutant channel of hTRPV1 (C157A) (Fig. 9, E and F). In this mutant, $[Ca^{2+}]_i$ responses to allicin were greatly reduced, but those to MS and MS-induced desensitization (data not shown) were unchanged. Taken together, these results suggest that MS activated and desensitized TRPV1 by interacting with sites distinct from those important for capsaicin and allicin.

**Methylsalicylate Activates Capsaicin-Sensitive Sensory Neurons of Rat.** Because we could not find evidence for an agonistic action of MS on endogenous hTRPV1 using human keratinocytes, we examined the effects of MS on cultured rat DRG neurons. In this study, cells were first stimulated with capsaicin (1 $\mu$M) to identify DRG neurons expressing TRPV1 and subsequently with MS (3 mM). Typ-
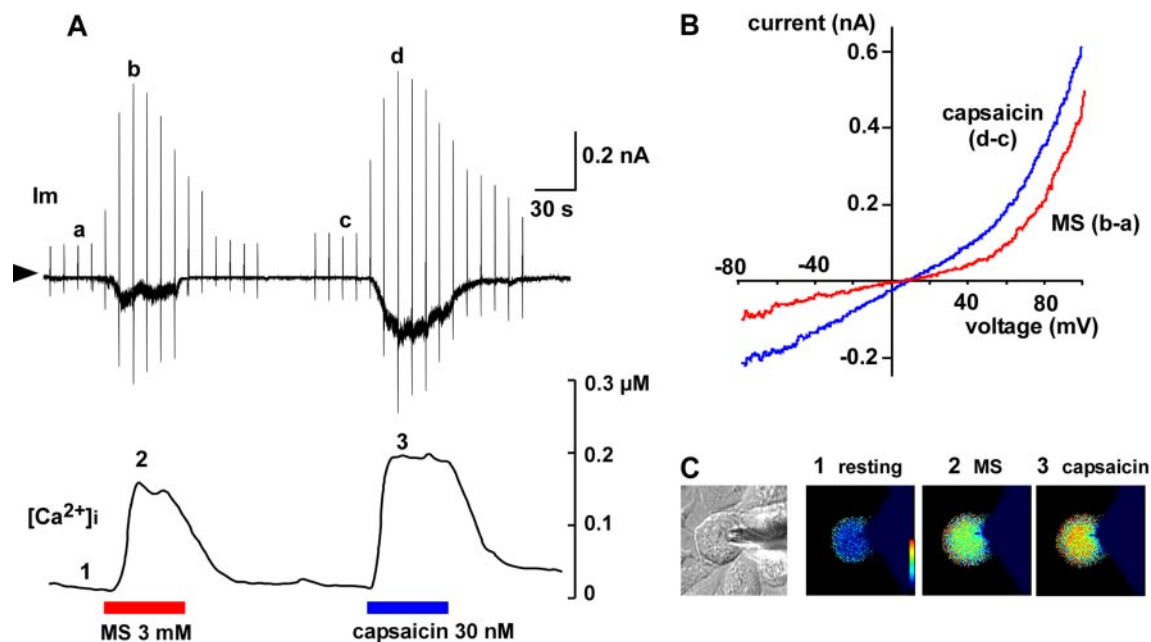
**Fig. 4.** Simultaneous measurement of current and $[Ca^{2+}]_i$ responses to methylsalicylate and capsaicin in the same human TRPV1-expressing HEK 293 cell. A, at a holding potential of $-60$ mV, MS (3 mM) or capsaicin (30 nM) was applied for 1 min. Before, during, and after these stimuli, ramp voltages from $-80$ to $+100$ mV for 100 ms every 10 s were applied at the points shown as vertical lines in the current trace (Im). The triangle beside the current shows 0 current level. B, current-voltage relationships induced by capsaicin and MS obtained with a ramp protocol are plotted as the difference from currents before their application (trace b minus a, trace d minus c). C, image under transmitted light of the patch-clamped cell. Pseudocolor time-lapse $Ca^{2+}$ images corresponding to the time points before (1, resting) and at the peak $[Ca^{2+}]_i$ responses induced by MS (2) and capsaicin (3). Similar data were obtained from five other hTRPV1-expressing cells.

ical and summarized $[Ca^{2+}]_i$ responses to MS and capsaicin in the same cells are shown in Fig. 10, A and B. Similar to the recombinant hTRPV1, MS produced a $[Ca^{2+}]_i$ increase in cells responding to capsaicin, and there was a positive relation between them ($r = 0.90$, $p < 0.01$; Fig. 10C). MS evoked increases of $[Ca^{2+}]_i$ in 201 of 268 DRG neurons, which were estimated to be neural cells based on the increase of $[Ca^{2+}]_i$ with 120 mM KCl. The majority of cells (94.5%) responding to MS also responded to capsaicin (190 of 201 neurons) and the remainder (11 neurons) were capsaicin-insensitive. The concentration-response relations for MS are depicted in Fig. 10D. Capsazepine reversibly suppressed the $[Ca^{2+}]_i$ response to MS in rat DRG neurons (Fig. 10E). These results indicated that MS had an agonistic action not only on recombinant TRPV1 channels but also on endogenous ones.

**Antinocifensive Action of Methylsalicylate.** To determine the clinical significance of the antinociceptive action of MS on TRPV1, we examined the effects of MS on capsaicin-induced nocifensive behavior in rats (Fig. 10F). Intraplantar injection of capsaicin-evoked nocifensive behavior ($23.2 \pm 2.7$ s, $n = 12$), which was significantly suppressed by the pretreatment with MS ($7.8 \pm 1.5$ s, $p < 0.05$). MS produced slight nocifensive responses just after its application ($3.3 \pm 0.7$ s).

## Discussion

MS is a naturally occurring compound that is used as an active ingredient of balms and liniments supplied as topical analgesics, especially as musculoskeletal pain relievers. Salicylate derivatives such as salicylic acid and acetylsalicylic acid (aspirin) are widely used for the treatment of inflammation and pain through the inhibition of COXs resulting in suppression of prostaglandin synthesis (Cashman and McAnulty, 1995; Wu, 1998; Vane and Botting, 2003). Despite the well known analgesic action of MS, the underlying molecular mechanisms independent of COX inhibition are not known. In the present experiment, we showed that MS was an effective agonist for recombinant human and porcine TRPV1. Moreover, MS rapidly desensitized TRPV1 channels than capsaicin and suppressed TRPV1 activation induced by poly-
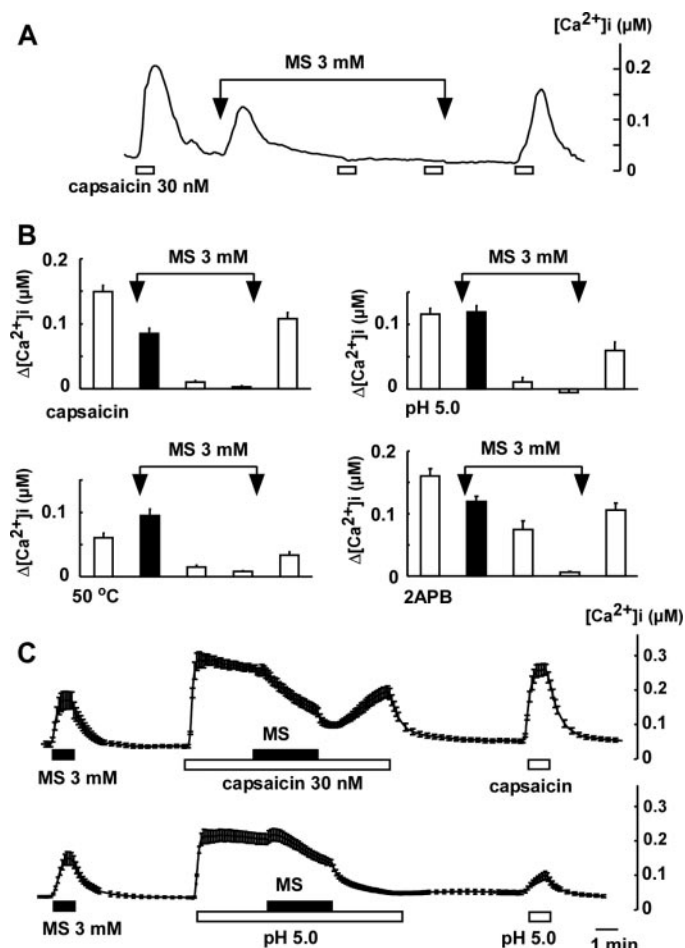


**Fig. 5.** Inhibitory effect of methylsalicylate on $[Ca^{2+}]_i$ responses to polymodal stimuli. A, representative trace showing MS-induced inhibition of $[Ca^{2+}]_i$ responses to capsaicin. Capsaicin (30 nM) was applied before, during, and after washout of MS (3 mM). B, summarized effects of polymodal stimuli-induced $[Ca^{2+}]_i$ increase in the absence and presence of MS. In each, open columns and the filled one show the magnitude of polymodal stimuli (capsaicin 30 nM, pH 5.0, 50°C, 2APB 0.1 mM)- and MS-induced increase of $[Ca^{2+}]_i$, respectively. These data were obtained using an experimental protocol similar to that described in A. C, averaged $[Ca^{2+}]_i$ responses to capsaicin (30 nM, top) and protons (pH 5.0, bottom) before and during the application of MS (3 mM). During the sustained $[Ca^{2+}]_i$ increase generated by capsaicin ($n = 42$) or pH 5.0 ($n = 35$), MS was added. Symbols with vertical lines show mean ± S.E.M.
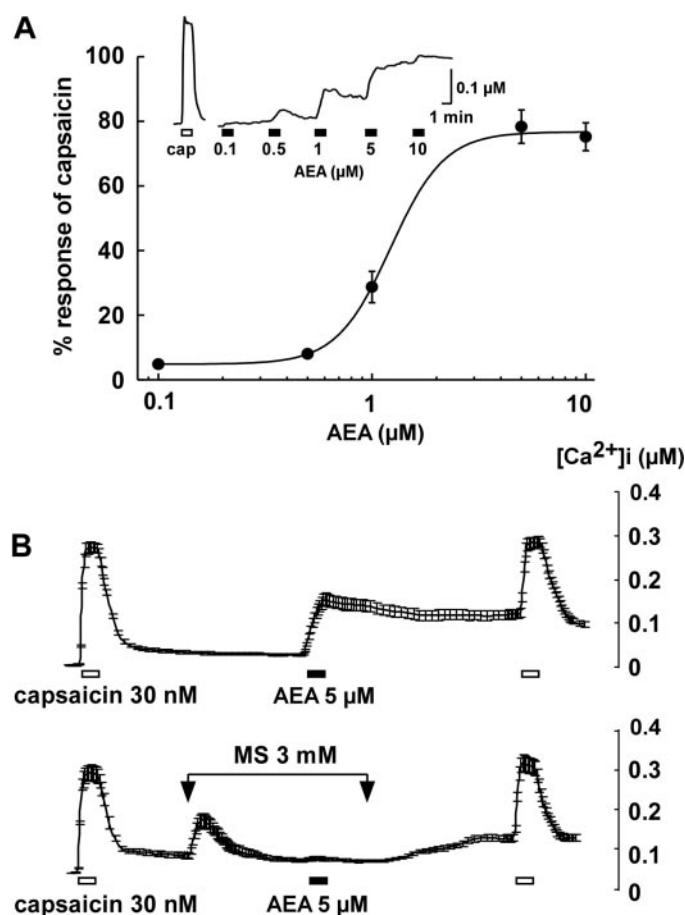


**Fig. 6.** Inhibition by methylsalicylate of TRPV1 activation by anandamide. A, concentration-response relationship for the change in $[Ca^{2+}]_i$ induced by anandamide (AEA, $n = 48$). Inset, representative traces of $[Ca^{2+}]_i$ responses to capsaicin (cap, 30 nM) and anandamide at various concentrations for 1 min. B, averaged $[Ca^{2+}]_i$ responses to capsaicin (30 nM) and anandamide (5 μM) in the absence (top trace, $n = 48$) and presence (bottom trace, $n = 40$) of MS (3 mM). Symbols with vertical lines show mean ± S.E.M.

modal stimuli. MS activated TRPV1 channels of rat sensory neurons in vitro and had antinocifensive action in vivo. Taken together, these results suggest that the MS-induced desensitization of TRPV1 is one of the underlying mechanisms of the analgesic action of MS.

Recent reports have demonstrated that MS is an agonist for TRPA1 (Bandell et al., 2004; Calixto et al., 2005), which forms $Ca^{2+}$-permeable nonselective cation channels as thermoreceptors sensitive to noxious cold (Story et al., 2003; Bandell et al., 2004). Regarding cold sensation, however, there is an ongoing debate over its potential role as a noxious cold sensor (Zurborg et al., 2007). In the present experiments, the possibility that hTRPV1 shared TRPA1 properties could

be excluded, because hTRPV1 did not respond to cinnamaldehyde and acrolein, which are known as agonists of TRPA1 (Bandell et al., 2004; Bautista et al., 2006). But allyl isothiocyanate (mustard oil) activated hTRPV1 similar to porcine TRPV1 as we reported previously (Ohta et al., 2007). Although the precise mechanisms by which MS activates TRPV1 are not clear, it is likely that MS directly binds to TRPV1 and promotes channel opening in a mechanism analogous to capsaicin activation (Jordt and Julius, 2002), because MS-induced responses occurred not only in intact cells but also in cells dialyzed with internal solution by the conventional whole-cell patch-clamped technique, of which configuration often results in the washout of internal signal
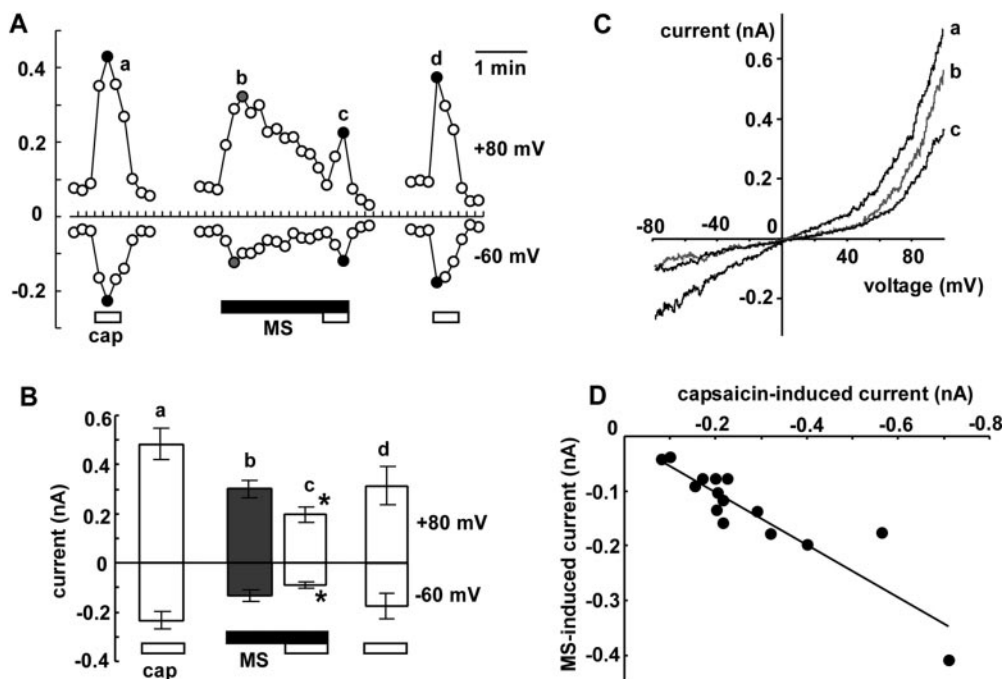


**Fig. 7.** Methylsalicylate inhibits current response to capsaicin. A, current responses to capsaicin (cap, 30 nM) were obtained before, during, and after washout of MS (3 mM). Ramp currents were evoked by 100-ms voltage ramps from $-80$ to $+100$ mV applied every 10 s. Symbols represent the current amplitude at $+80$ and $-60$ mV. B, summarized current amplitudes of capsaicin with and without MS at $+80$ and $-60$ mV. The open columns and filled one show the current amplitude induced by capsaicin and MS, respectively. ∗, $p < 0.05$ versus the response before application of MS. Symbols with vertical lines show the mean $\pm$ S.E.M. ($n = 11$). C, current-voltage relationship obtained with the ramp protocol induced by capsaicin before MS application (a), by MS (b), and capsaicin (c) during MS application. D, relationship between the amplitudes of capsaicin- and MS-induced inward current at $-60$ mV in individual cells ($r = 0.89$, $p < 0.01$, $n = 15$).
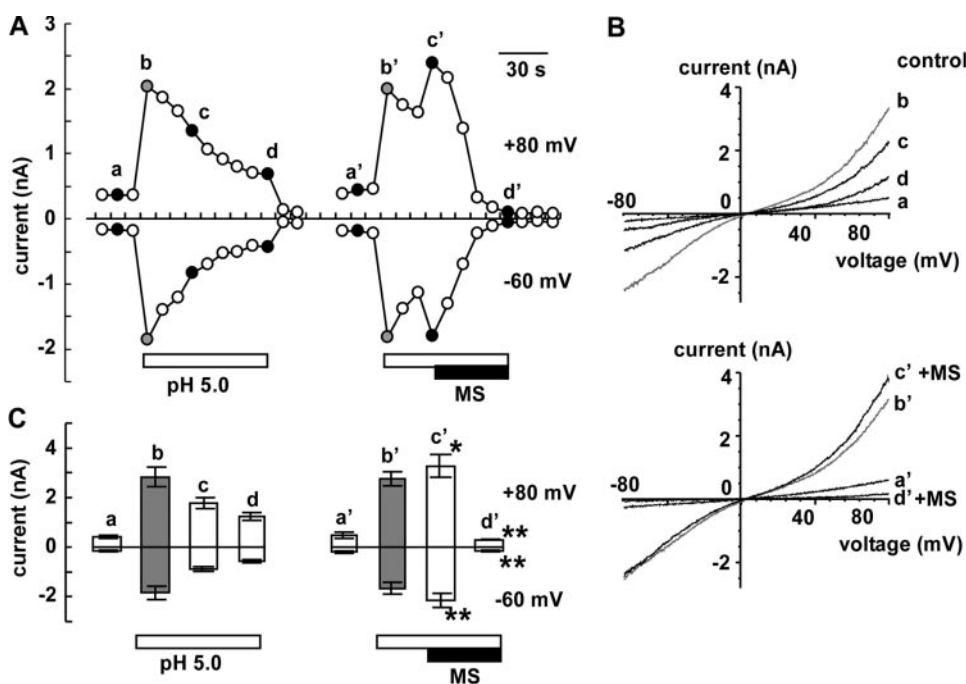


**Fig. 8.** Methylsalicylate inhibits current responses to protons. A, ramp currents were evoked by 100-ms voltage ramps from $-80$ to $+100$ mV applied every 10 s. Each symbol represents the current amplitude at $-60$ and $+80$ mV in the same cell. Left, control current response to pH 5.0. Right, MS (3 mM) was applied after development of proton-induced current. B, current-voltage relations obtained with the ramp protocol are plotted at time points alphabetically labeled corresponding to A. C, summarized current amplitudes of protons before (a), and at 10 (b), 40 (c), and 90 s (d) after the application of protons. Time-matched effects of MS on current responses before (a′), and at 10 (b′), 40 (c′), and 90 s (d′) after the application of protons. Left, control responses to protons; right, proton-induced currents before and after the addition of MS. Symbols with vertical lines show mean $\pm$ S.E.M. ∗, $p < 0.05$; ∗∗, $p < 0.01$ versus time-matched control (c versus c′, d versus d′).

molecules (Horn and Marty, 1988). A recent report showed that the activation of TRPV1 by the local anesthetic lidocaine required the presence of $PIP_2$ (Leffler et al., 2008). However, this is unlikely to be the case for the MS effects, because the MS-induced $[Ca^{2+}]_i$ increase was not affected by pretreatment with a high concentration of wortmannin. It has been reported that high concentrations of wortmannin inhibit resynthesis of $PIP_2$ through phosphatidylinositol 4-kinase, which in turn leads to the depletion of the cellular $PIP_2$ level (Suh and Hille, 2002; Liu et al., 2005). In HEK 293 cells stably expressing rat TRPV1, the treatment with wortmannin potentiates the capsaicin-induced $[Ca^{2+}]_i$ increase (Vetter et al., 2008). In our study, however, wortmannin treatment did not influence the capsaicin-induced $[Ca^{2+}]_i$ responses. This may be due to different endogenous $PIP_2$ levels in the cells used for transfection, species differences of transfected TRPV1 or the conditions of transfection.

$[Ca^{2+}]_i$ and current responses to MS were smaller than those to 30 nM capsaicin or pH 5.0 (Figs. 2 and 7). Thus, we suggest that for TRPV1, MS has both low intrinsic efficacy and potency, in some ways resembling that of camphor (Xu et al., 2005) and mustard oil (Ohta et al., 2007). Although the concentrations of MS required to activate TRPV1 are high, the activation is likely to be physiologically relevant, because

MS-containing balms are used in topical preparations at 2 to 20%, which corresponds to 0.1 to 1 M. In the present study, we could not confirm MS actions on native human TRPV1 channels. It has been reported that TRPV1-like immunoreactivity is observed in human skin biopsies (Denda et al., 2001), and capsaicin evokes a $[Ca^{2+}]_i$ increase in human keratinocytes in culture (Inoue et al., 2002). However, no $[Ca^{2+}]_i$ response was induced by capsaicin even at a high concentration (0.1 mM), but camphor and 2APB evoked elevation of $[Ca^{2+}]_i$. This was due to keratinocytes expressing TRPV3 (Peier et al., 2002), which is activated by camphor and 2APB (Chung et al., 2004; Moqrich et al., 2005). We suggest that not all human keratinocytes express TRPV1, or there might be cell line-dependence. Nevertheless, we succeeded in finding evidence that MS had a stimulatory effect on endogenous TRPV1 channels using cultured rat sensory neurons. MS evoked a $[Ca^{2+}]_i$ increase in dorsal root ganglion neurons mainly responding to capsaicin, and the MS-induced $[Ca^{2+}]_i$ increase was reversibly suppressed by capsazepine. A stimulatory effect of MS was also observed in HEK 293 cells heterologously expressing porcine TRPV1. Therefore, there may be little species difference in the action of MS on TRPV1, unlike capsaicin (Jordt and Julius, 2002;
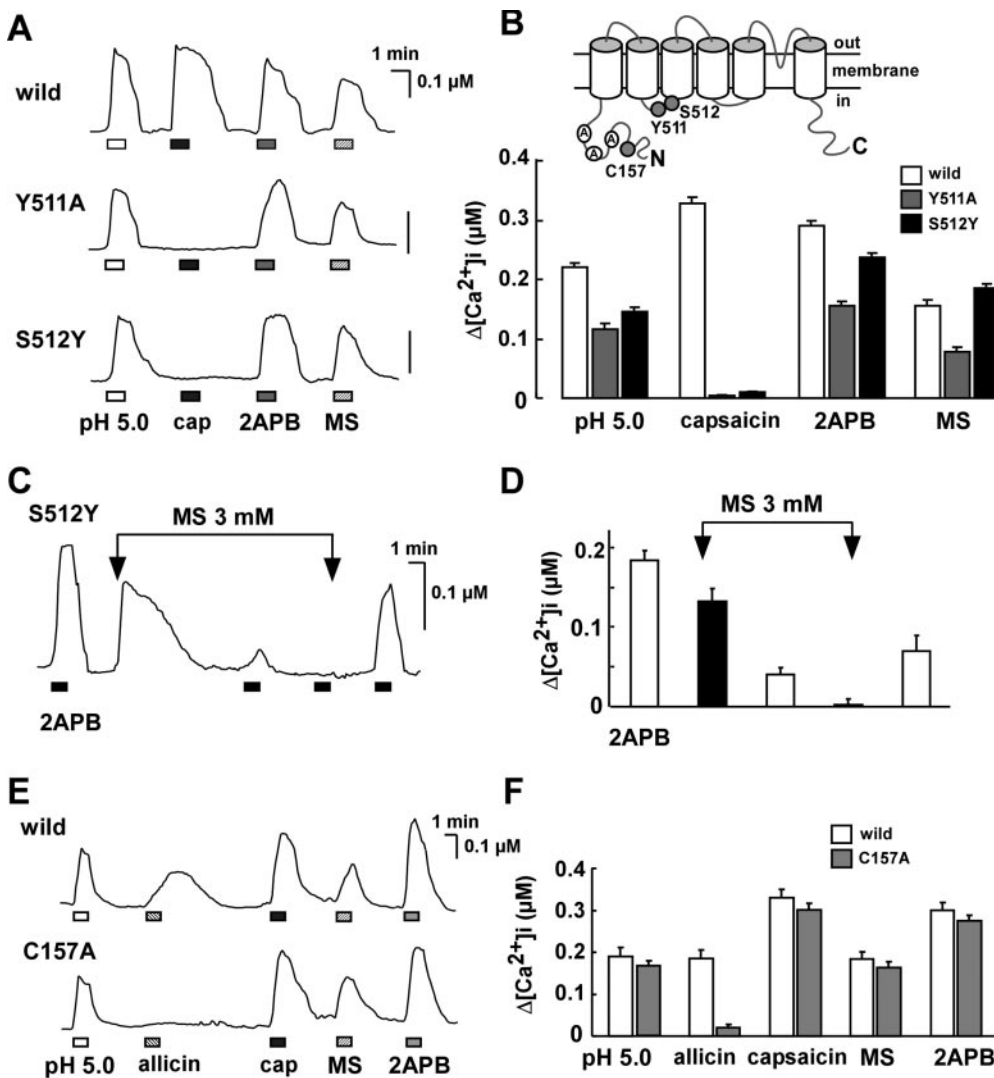


**Fig. 9.** Methylsalicylate activates TRPV1 via amino acids independent of capsaicin and allicin sensitivity. A, representative $[Ca^{2+}]_i$ responses to pH 5.0, capsaicin (cap, 1 $\mu$M), 2APB (0.1 mM), and MS (3 mM) in wild and two mutant channels. Capsaicin-insensitive point mutants of hTRPV1 (Y511A and S512Y) showed no effect of capsaicin but reacted to other stimuli. B, illustration representing the topological structure of the TRPV1 receptor. Barrels show the putative transmembrane regions, and the circles indicate the proposed locations of the cysteine (Cys157) at a position responsible for allicin sensitivity and tyrosine (Tyr511) and serine (Ser512) residues at positions responsible for vanilloid sensitivity. A,; ankyrin repeat. Summarized changes in $[Ca^{2+}]_i$ induced by protons, capsaicin, 2APB, and MS in wild ($n = 55$), Y511A ($n = 60$), and S512Y channels ($n = 51$). C and D, representative and summarized $[Ca^{2+}]_i$ responses to 2APB before, during, and after the application of MS in a capsaicin-insensitive point mutant (S512Y). Symbols with vertical lines show mean $\pm$ S.E.M. ($n = 40$). E, representative $[Ca^{2+}]_i$ responses to pH 5.0, allicin (0.1 mM), capsaicin (cap, 0.3 $\mu$M), MS (3 mM), and 2APB (0.1 mM) in wild and allicin-insensitive mutant channels (C157A). F, summarized changes in $[Ca^{2+}]_i$ induced by protons, allicin, capsaicin, MS, and 2APB in wild ($n = 50$) and C157A channels ($n = 62$).

Gavva et al., 2004) and phorbol 12-phenylacetate 13-acetate 20-homovanillate (Phillips et al., 2004).

TRPV1 exhibits desensitization, which is a diminished response during constant agonist application (Koplas et al., 1997). The desensitization of TRPV1 probably contributes to the analgesic effects of capsaicin (Szolcsányi, 2004). In the present study, TRPV1 activation of MS exhibited a marked decrease despite the continued presence of MS. In the presence of MS, not only the $[Ca^{2+}]_i$ response to capsaicin but also those to heat, protons, 2APB, and anandamide were suppressed. Furthermore, MS inhibited capsaicin- and proton-induced current responses under voltage-clamped conditions. It has been reported that TRPV1 desensitization depends on $Ca^{2+}$-dependent (described later) and $Ca^{2+}$-independent mechanisms (Koplas et al., 1997; Tominaga and Tominaga, 2005). Because MS-induced TRPV1 desensitization was ob-

served under patch-clamp conditions with dialysis by a high concentration of the $Ca^{2+}$ chelator EGTA, it is likely that a primary desensitizing mechanism was $Ca^{2+}$-independent. The mechanism of TRPV1 suppression by MS is not clear. It seems unlikely that MS-induced TRPV1 block occurs via partial agonism, because MS inhibited polymodal activation of TRPV1. Perhaps the inhibitory action of MS might be mediated through some physiochemical interaction with TRPV1 channels.

MS-induced TRPV1 activation and the following desensitization were suggested to be related to the analgesic and counterirritant properties of MS. In rats, MS suppressed nocifensive behavior induced by intraplantar capsaicin, which is mediated via TRPV1 activation (Caterina et al., 2000). A similar desensitizing effect was reported for camphor on TRPV1 channels (Xu et al., 2005).

The location of the putative MS-responsive site on TRPV1 is not clear. Most chemical agonists of TRPV1 share structure similarity, often containing a vanilloid moiety, and activate TRPV1 via similar mechanisms (Jordt and Julius, 2002). Although MS does not contain a vanilloid group, it is quite hydrophobic and could plausibly interact with TRPV1 channels at the same locus. However, MS activation persisted in point mutants of hTRPV1 (Y511A and S512Y), which were reported to nearly abolish capsaicin activation (Jordt and Julius, 2002; Sutton et al., 2005), and this was confirmed in the present study. The effects of 2APB, a common agonist for TRPV1, TRPV2, and TRPV3 (Hu et al., 2004) could activate these mutant channels, suggesting that these vanilloid recognition sites were not related to 2APB action. Similar to our data, camphor can activate TRPV1 in some capsaicin-insensitive mutant channels (Xu et al., 2005). In contrast to the present MS effect, however, the stimulatory effect of camphor is not influenced by capsazepine (Xu et al., 2005). Therefore, the modes of action of MS and camphor may be different. These results suggest that MS activates hTRPV1 by interacting with channel regions distinct from those important for capsaicin and camphor. Furthermore, MS could activate C157A channels, which are insensitive to allicin (Salazar et al., 2008). Moreover, a large amount of $Ca^{2+}$ produces TRPV1 desensitization, which leads to a decrease of the apparent affinity for capsaicin (Vyklický et al., 2009). This seems to be the case in the present results for capsaicin-induced $[Ca^{2+}]_i$ responses, but no obvious desensitization of MS-induced $[Ca^{2+}]_i$ responses occurred (Fig. 2E). From this desensitization result and mutant experiments, it seems likely that MS activates TRPV1 at a novel binding site or mechanism. The target amino acids of TRPV1 responsible for the action of MS remain to be determined.

Apart from the direct action of MS on TRPV1, indirect antinociceptive and anti-inflammatory actions of MS have been suggested (Zhang et al., 2007). Gaultherin, a methylsalicylate diglycoside, which is the major constituent of gaultherin isolated from *Gaultheria yunnanesis,* has been reported to show analgesic effects on acetic acid-induced writhing and formalin-induced pain in mice and rats (Calvo, 2006; Zhang et al., 2006). This compound is suggested to be metabolically converted to salicylate, resulting in the inhibition of the COX activity that synthesizes prostaglandin $E_2$. In the present study, MS-induced TRPV1 activation was not affected by indomethacin, a nonselective COX inhibitor (data not shown). It has been reported that acid-sensing ion chan-
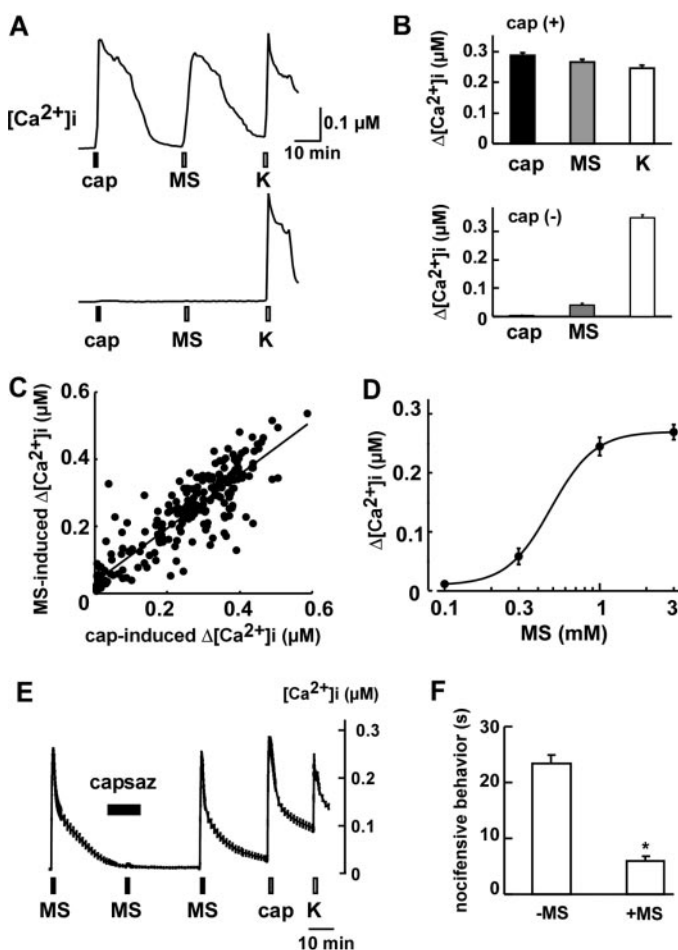


**Fig. 10.** MS stimulates endogenous TRPV1 in cultured rat dorsal root ganglion neurons and inhibits capsaicin-induced nocifensive behavior in rats. A, cells were sequentially challenged with capsaicin (cap, 1 $\mu$M), MS (3 mM), and KCl (K, 120 mM) for 1 min at intervals of 30 min. Top and bottom traces show representative $[Ca^{2+}]_i$ responses in cells responding and not responding to capsaicin, respectively. B, summarized amplitude of $[Ca^{2+}]_i$ in capsaicin-sensitive (cap+, $n = 201$) and -insensitive neurons (cap−, $n = 67$). C, relationship between the amplitudes of MS- and capsaicin-induced $[Ca^{2+}]_i$ increases in rat DRG neurons ($n = 201$). D, concentration-response relationship for the change in $[Ca^{2+}]_i$ responses to MS ($n = 45$). E, capsazepine (capsaz, 1 $\mu$M) reversibly suppressed $[Ca^{2+}]_i$ response to MS ($n = 34$) in rat DRG neurons. F, MS (0.5 mg/paw) reduced nocifensive behavior induced by intraplantar injection of capsaicin (0.5 $\mu$g/paw, $n = 12$). ∗, $p < 0.05$. Symbols with vertical lines show mean ± S.E.M.

nels are targets for the analgesic actions of nonsteroidal anti-inflammatory drugs through the inhibition of acid-sensing ion channel activity (Voilley, 2004). Thus, both direct and indirect actions of MS may be involved in its antinociceptive action in vivo.

It is now well recognized that TRPV1 is an important molecular gateway to the pain sensation (Szallasi and Blumberg, 1999). Various naturally occurring substances have been considered as possible candidates targeting TRPV1 channels (Calixto et al., 2005). Because we have demonstrated that MS has stimulatory and inhibitory effects on TRPV1 channels, the present experiment may provide new information for the development of therapeutic and analgesic drugs targeted at TRPV1.

### Acknowledgments

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