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# Neurokinin 2 receptor-mediated activation of protein kinase C modulates capsaicin responses in DRG neurons from adult rats

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## Abstract

Patch-clamp techniques and $Ca^{2+}$ imaging were used to examine the interaction between neurokinins (NK) and the capsaicin (CAPS)-evoked transient receptor potential vanilloid receptor 1 (TRPV1) responses in rat dorsal root ganglia neurons. Substance P (SP; 0.2–0.5 μM) prevented the reduction of $Ca^{2+}$ transients (tachyphylaxis) evoked by repeated brief applications of CAPS (0.5 μM). Currents elicited by CAPS were increased in amplitude and desensitized more slowly after administration of SP or a selective $NK_2$ agonist, [$Ala^8$]-neurokinin A (4–10) (NKA). Neither an $NK_1$-selective agonist, [$Sar^9$, $Met^{11}$]-SP, nor an $NK_3$-selective agonist, [$MePhe^7$]-NKB, altered the CAPS currents. The effects of SP on CAPS currents were inhibited by a selective $NK_2$ antagonist, MEN 10,376, but were unaffected by the $NK_3$ antagonist, SB 235,375. Phorbol 12,13-dibutyrate (PDBu), an activator of protein kinase C (PKC), also increased the amplitude and slowed the desensitization of CAPS responses. Phosphatase inhibitors, decamethrin and α-naphthyl acid phosphate (NAcPh), also enhanced the currents and slowed desensitization of CAPS currents. Facilitatory effects of SP, NKA and PDBu were reversed by bisindolylmaleimide, a PKC inhibitor, and gradually decreased in magnitude when the agents were administered at increasing intervals after CAPS application. The decrease was partially prevented by prior application of NAcPh. These data suggest that activation of $NK_2$ receptors in afferent neurons leads to PKC-induced phosphorylation of TRPV1, resulting in sensitization of CAPS-evoked currents and slower desensitization. Thus, activation of $NK_2$ autoreceptors by NKs released from the peripheral afferent terminals or by mast cells during inflammatory responses may be a mechanism that sensitizes TRPV1 channels and enhances afferent excitability.

### Keywords

capsaicin; desensitization; dorsal root ganglia; nociception; phosphorylation; transient receptor potential vanilloid receptor 1

## Introduction

Pain is initiated when a subgroup of small sensory neurons is activated by noxious chemical, mechanical or thermal stimuli (Caterina *et al.*, 1997; Gschossmann *et al.*, 2000; Caterina & Julius, 2001). Some of these neurons express the transient receptor potential vanilloid receptor 1 (TRPV1), which is a temperature- and acid-sensing receptor that also responds to capsaicin (CAPS), and is thought to play an important role in the activation of sensory nerves by noxious stimuli (Caterina & Julius, 2001; Montell *et al.*, 2002). Stimulation of TRPV1 with CAPS induces afferent firing and triggers the release of neuropeptides, including substance P (SP)


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and neurokinin A from central and peripheral afferent nerve terminals (Saban *et al.*, 1997; Caterina & Julius, 2001). The demonstration that TRPV1 channel blockers attenuate certain types of pain behavior in animals (Woolf & Mannion, 1999; Ghilardi *et al.*, 2005) raises the possibility that these agents might be useful clinically in treating pain (Westaway, 2007).

The present study evaluated the modulation of TRPV1 channels by afferent neuropeptides (i.e. SP, NKs). In addition to acting on the effector organs and on the second order neurons in the spinal cord, afferent neuropeptides are also thought to act in an autofeedback manner on the G-protein-coupled receptors present on the afferent terminals and thereby modulate afferent excitability and transmitter release (Malcangio & Bowery, 1999; Morrison *et al.*, 1999; Rathee *et al.*, 2002). Dorsal root ganglion (DRG) neurons in primary cultures express all three NK receptors (Brechenmacher *et al.*, 1998), and NKs are known to modulate voltage-gated $Ca^{2+}$ channels in sensory neurons by activating protein kinase C (PKCε; Sculptoreanu & de Groat, 2003). One type of protein kinase, PKCε (Numazaki *et al.*, 2002; Zhou *et al.*, 2003; Mandadi *et al.*, 2004), has been implicated in nociceptive sensitization and thermal hyperalgesia (Bhave *et al.*, 2003; Varga *et al.*, 2006). Activation of PKCε potentiates heat-evoked currents and lowers the threshold for the temperature-activated TRPV1 currents (Cesare *et al.*, 1999), whereas deletion of PKCε attenuates thermal- and acid-induced hyperalgesia (Khasar *et al.*, 1999).

TRPV1 receptors can be turned off in the continuous presence of CAPS (acute desensitization) or during repeated brief applications of CAPS (tachyphylaxis; Koplas *et al.*, 1997). This property is responsible for the transient nature of the CAPS currents. Because the time course of desensitization is relatively slow, various drugs can be tested during the partial desensitization phase of CAPS currents (Koplas *et al.*, 1997; Sculptoreanu *et al.*, 2005b). Desensitization is prevented by PKC phosphorylation of both serine 502 and serine 800 in TRPV1 (Mandadi *et al.*, 2004), whereas dephosphorylation mediated by a $Ca^{2+}$ -dependent phosphatase, calcineurin, is thought to be involved in desensitization (Koplas *et al.*, 1997; Piper *et al.*, 1999). Thus, TRPV1 is regulated by a phosphorylation–dephosphorylation cycle that requires PKC and calcineurin.

In the present experiments, we examined the possibility that SP and related peptides might modulate TRPV1 via a PKC-mediated intracellular signaling pathway in dissociated DRG neurons of adult rat. The results suggest that activation of $NK_2$ receptors enhances CAPS responses and slows the desensitization that occurs with prolonged application of CAPS. The changes in CAPS-evoked responses induced by NKs were antagonized by a PKC inhibitor, suggesting that they require activation of PKC.

## Materials and methods

### Experimental animals

Experiments were performed on adult male Sprague–Dawley rats (200–250 g; Harlan, Indianapolis, IN, USA). Care and handling of the animals have been approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

### Cell culture

For patch-clamp experiments, neurons were isolated from L4–S3 DRG of adult male rats using methods previously described (Sculptoreanu & de Groat, 2003). Surgery was performed under halothane anesthesia. After spinal cord dissection animals were killed by cervical dislocation. Briefly, freshly removed ganglia were minced and enzymatically digested at 37 °C for 10 min in Dulbecco's modified essential medium (DMEM) containing 2.5 mg/mL trypsin, followed by 50 min in DMEM containing 2.5 mg/mL collagenase D (Boehringer-Mannheim) and 4 mg/

mL trypsin inhibitor type 2S (Sigma). The ganglia were then dissociated mechanically by titration using siliconized Pasteur pipettes. The cell suspension was layered on DMEM containing 50% bovine serum albumin (BSA) and centrifuged at 112 ***g*** at room temperature to remove most of the debris and broken cells. After withdrawing the supernatant, the pellet containing neurons was resuspended in DMEM containing 10% heat-inactivated horse serum and 5% fetal BSA, and plated on collagen-coated 35-mm Petri dishes (Biocoat; Becton Dickinson) and kept in 95% air and 5% $CO_2$ incubators at 37 °C until recording.

For $Ca^{2+}$ imaging, L6 and S1 DRGs were removed by laminectomy from halothane-anesthetized rats. DRGs were placed in cold oxygenated DMEM, washed to remove blood and minced. Tissue was incubated in a cocktail of trypsin (type 1, Sigma, 0.3 mg/mL), collagenase (type 1, Sigma, 1 mg/mL) and DNAase (type 4, Sigma, 0.5 mg/mL) in 5 mL DMEM at 35 °C in a shaking water bath for 25–30 min. Trypsin inhibitor (type 2a, Sigma, 3 mg/mL) was then added and cells were triturated several times using fire-polished Pasteur pipettes with decreasing diameters, until free cells were observed. Cells were centrifuged for 5 min at 112 ***g***, the supernatant was removed and replaced with DMEM supplemented with 10% horse serum and 7.5% fetal bovine albumin. Cells were plated on collagen-coated glass coverslips. Media was added 2 h after incubation at 37 °C. Cells were used within 2–24 h after dissociation.

## Drugs

CAPS; the phorbol ester, phorbol 12,13-dibutyrate (PDBu); the phosphatase inhibitors α-naphthyl acid phosphate (NAcPh) and decamethrin (DEC); the PKC inhibitor bisindolylmaleimide (BIM); SP; $NK_1$ agonist [$Sar^9$, $Met^{11}$]-substance P (SarMetSP); $NK_2$ agonist NKA; $NK_3$ agonist [$MePhe^7$]-neurokinin B (NKB) were obtained from Calbiochem. An $NK_2$ antagonist, MEN 10,376, and adenosine-5′-O-(3-thiotriphosphate) (ATPγS) were obtained from Sigma. The $NK_3$ receptor antagonist SB 235,375 was a gift from SmithKline Beecham. The TRPV1 antagonist (diaryl piperazine) was a gift from Neurogen.

## Current-clamp and voltage-clamp recording

Gigaohm-seal whole-cell recordings of CAPS currents were recorded in DRG neurons after 2–5 days in culture using whole-cell patch-clamp techniques. Neuronal processes that appear after 5 days in culture may significantly contribute to a slowly decaying capacitative current. Neurons from cultures older than 5 days in which the slowly decaying currents become significant were excluded in our analysis. Patch pipettes were pulled from capillary glass tubes (Accufil 90, Clay-Adams) and fire polished. Immediately before recording, the serum-containing media was replaced with phosphate-buffered saline (Invitrogen) of the following composition (in mM): NaCl, 138; KCl, 2.6; $CaCl_2$, 0.9; $MgCl_2$, 0.5; $KH_2PO_4$, 1.5; $Na_2$ $HPO_4$, 8.1; pH 7.2. Action potentials in response to current injections were recorded using an Axopatch 200A (Axon Instruments, Foster City, CA, USA) amplifier. Pulse generation, membrane potential recording and data analysis used pClamp software (Axon Instruments). Voltage changes were sampled at 50–500-ls intervals and filtered at 2–10 kHz. Action potentials recorded in current-clamp mode were generated by rectangular current-pulse injections 5 ms long and 50–500 pA in intensity, followed by a 100-ms interpulse at the holding potential and a second pulse, 600 ms long. Whole-cell CAPS currents were recorded in voltage-clamp mode. Currents activated by CAPS or NKs were sampled at 5 kHz, and the high-frequency noise was filtered at 500 Hz post-recording (in Clampfit, using a Gaussian filter). Capacitive currents and up to 80% of the series resistance were compensated.

The pipette (intracellular) solution contained (in mM): KCl, 120; $K_2HPO_4$, 10; NaCl, 10; $MgCl_2$, 2; EGTA, 1; HEPES, 10; pH adjusted to 7.4 with HCl. To this solution Mg-ATP (3 mM), cAMP (0.3 mM) and Tris-GTP (0.5 mM) were added just prior to the experiments. CAPS, the TRPV1 antagonist, PDBu, NAcPh, DEC and BIM were dissolved in dimethylsulfoxide

(DMSO; 100 mM) and used at less than 0.01% of their stock concentration. At these dilutions, DMSO alone had no effect on TRPV1 responses to CAPS. Stock solutions (10–100 mM) were stored at −20 °C and diluted in the external recording solution just before experiments. The TRPV1 antagonist (diaryl piperazine) was shown previously to be a potent and selective TRPV1 inhibitor ($K_i$ = 6 nM for inhibition of acid-evoked responses and 35 nM for inhibition of CAPS-evoked responses; Valenzano *et al.*, 2003). The following NK agonists and antagonists were prepared in aqueous solutions: SP, Sar,MetSP, NKA, NKB, MEN 10,376 and SB 235,375. The $NK_2$-selective agonist used in our study (NKA) was a highly potent ($EC_{50}$ of $4.8 \times 10^{-9}$ M) and selective agonist for human recombinant $NK_2$ receptors expressed in Chinese hamster ovary cells (Subramanian *et al.*, 1994). In the same cells, the $NK_3$ (NKB)- and $NK_1$ (SarMetSP)-selective agonists also used in our study had no effects. On the other hand, the $NK_1$-selective agonist used in our study was a highly potent agonist for $NK_1$ receptors in salivary glands (Giuliani *et al.*, 1988). In these cells the $NK_2$ and $NK_3$ receptor agonists used in our studies had no effects on $NK_1$ receptors expressed in the salivary glands (Giuliani *et al.*, 1988). Extracellularly applied drugs were pipetted from stock solutions at 10–100 × the final bath concentration and rapidly mixed in the recording chamber as described previously (Sculptoreanu & de Groat, 2003).

### Patch-clamp data analysis

We compared responses to agents applied either prior to CAPS or after partial desensitization of CAPS responses. The initial rapid decay of TRPV1 currents in response to continued application of CAPS and after addition of other agents (PDBu, NKs or BIM) in the presence of CAPS was fitted with single exponentials using pClamp software regression analysis for simple exponential decay. Whole-cell currents evoked by CAPS alone were normalized to membrane capacitance in each cell, averaged and fitted with an equation that contained one exponential for the rising phase and two exponentials for the decaying phase of the form: $y = f_1 + f_2$; where $f_1$ is the rising phase [$f_1 = a^* (1-\exp(-(x-l_1)/\tau_r)) + c_1$] and $f_2$ is the decaying phase of the response [$f_2 = b^*\exp(-(x - l_2)/\tau_f) + d^*\exp(-(x - l_3)/\tau_s)$]; where $x$ is time, $l_1$–$l_3$ lag times (time between application of drug and measurable response), and $\tau_r$, $\tau_f$ and $\tau_s$, rise time constant of current activation and the fast and slowly decaying time constants of the current desensitization. These equations were fitted to the data using non-linear regression analysis in the Sigma Plot program.

### Statistical analysis

Results are reported as mean ± SEM. Statistical testing for the data presented in Figs 1F, 2D and E, 3D and E, and 5B and C was carried out using a stepwise procedure depending upon the number of groups being compared. When only two means were involved in a comparison, a two-tailed *t*-test with unequal variances was used. A comparison was considered statistically significant if $P < 0.05$. When more than two means were involved, a one-way analysis of variance was first carried out in order to obtain a global test of the null hypothesis. If the global *P*-value for the test of the null hypothesis was < 0.05, we carried out *post hoc* comparisons between the different groups using the Holm–Sidak test (Glantz, 2005). The global *P*-value for these comparisons was used to determine statistical significance.

### $Ca^{2+}$ imaging

DRG cells were loaded with fura-2 AM (2 μM; Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C in an atmosphere of 5% $CO_2$. Fura-2 AM was dissolved in the bath solution [Hank's balanced salt solution (HBSS)] containing (in mM): NaCl, 138; KCl, 5; $KH_2PO_4$, 0.3; $NaHCO_3$, 4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; glucose, 5.6; pH 7.4, 310 mOsm/L, to which BSA was added (5 mg/mL; Sigma, St Louis, MO, USA) to promote loading. Fura-2 $Ca^{2+}$ imaging was performed as previously described (Ene *et al.*, 2003). Coverslips were placed on

an inverted epifluorescence microscope (Olympus IX70) and continuously superfused with HBSS. Fura-2 was excited alternately with UV light at 340 and 380 nm, and the fluorescence emission detected at 510 nm using a computer-controlled monochromator. Image pairs were acquired every 1–30 s using illumination periods between 20 and 50 ms. Wavelength selection, timing of excitation and the acquisition of images were controlled using the program C-Imaging (Compix, PA, USA) running on a PC. Digital images were stored on hard disk for off-line analysis.

Drugs were dissolved in external solution from concentrated stock solutions and delivered via bath application using a gravity-driven application system. Stock solution of CAPS (10 mM) was made in 10% ethanol, 10% Tween 80 and 80% physiological saline. The vehicle used for dissolving CAPS did not produce an increase in the intracellular calcium concentration when applied alone.

Image analysis was performed using the program C-Imaging (Compix, Cranberry Township, PA, USA). Background was subtracted to minimize camera dark noise and tissue auto-fluorescence. An area of interest was drawn around each cell and the average value of all pixels included in this area was taken as one measurement. Baseline intracellular $Ca^{2+}$ concentration was determined from the average of five to eight measurements obtained before drug application. Amplitudes of peak $Ca^{2+}$-responses were computed as the difference between the peak value and the baseline value. In order to be considered as drug-induced responses, changes in intracellular $Ca^{2+}$ concentration had to occur immediately (1–10 s) following stimulation and the amplitudes had to exceed baseline by 2 standard deviations. FAU stands for fluorescence arbitrary units and R is ratio of fluorescence signal measured at 340 nm divided by the fluorescence signal measured at 380 nm. The results are given as changes in R before and after drug application, and as a percentage increase of R above resting levels of intracellular $Ca^{2+}$ concentration. Data analysis was performed using Excel (Microsoft, Redmond, WA, USA) and Origin (OriginLab, Northampton, MA, USA). Statistical significance was tested using paired *t*-test and ANOVA followed by Tukey–Kramer *post hoc* test. Throughout the text data are given as mean ± SEM.

## Results

### Properties of DRG neurons

Experiments were conducted on 140 lumbosacral (L4–S3) DRG neurons, of which 83 (59%) responded to a submaximal CAPS concentration (0.5 μM). Previous studies in rat DRG neurons revealed that the $ED_{50}$ for CAPS is 0.7 μM (Koplas *et al.*, 1997; Vellani *et al.*, 2001). CAPS (0.5 μM) elicited inward currents ranging from 50 pA to 10 nA in amplitude, averaging −958 ± 190 pA in amplitude (average current densities of 11 ± 2 pA/pF, $n = 61$), which desensitized in the continuous presence of CAPS (Fig. 1). The CAPS-responsive cells studied in our experiments ranged from 30 to 50 μm in diameter and had an average membrane capacitance of 58 ± 4 pF, resting membrane potential of −53 ± 2 mV and membrane resistance of 224 ± 15 MΩ. In these neurons, when held at −57 mV membrane potential, rectangular pulses 50–500 pA in intensity and 600 ms in duration elicited only a small number of action potentials (range 1–4 APs/600 ms, average 2.9 ± 0.4 APs/600 ms), as reported previously for CAPS-responsive DRG neurons (Sculptoreanu & de Groat, 2007; Yamane *et al.*, 2007).

### Agonist-evoked currents

The currents elicited in response to CAPS (0.5 μM; Figs 1A–C and 2A–D, dashed lines) typically reached a peak at 31 ± 3 s after CAPS application and decayed thereafter. Application of SP (Fig. 1C and G), NKA (an $NK_2$-selective agonist; Fig. 1D and G), NKB (an $NK_3$-selective agonist; Fig. 1G) or SarMetSP (an $NK_1$-selective agonist, not shown) in a concentration (0.5

μM) reported previously to produce near-maximal enhancements of voltage-dependent $Ca^{2+}$ currents in DRG neurons (Sculptoreanu & de Groat, 2003) evoked rapidly activating currents that were a quarter or less in magnitude than CAPS currents and fully desensitized in less than 1 min.

The activation of CAPS currents lagged the application of CAPS by 6.6 ± 0.4 s (Fig. 1B and F). This lag was considerably longer than that of ATPγS-evoked currents (50 μM), which in the same neurons had an average lag time of 1.9 ± 0.7 s (Fig. 1F). NK-evoked inward currents, mediated by activation of an unidentified non-selective cation channel (Yang *et al.*, 2003), had lag times comparable to those of CAPS currents (Fig. 1F and G): SP, 6.4 ± 0.3 s; NKA, 6.2 ± 0.5 s; and NKB, 6.7 ± 1.2 s. On the other hand, PDBu (0.5 μM) alone did not elicit a membrane current (Fig. 1E). The rapid onset, rapid desensitizing currents evoked by the NK-selective agonists suggest that all subtypes of NK receptors ($NK_1$, $NK_2$ and $NK_3$) are expressed in DRG neurons (present work; Brechenmacher *et al.*, 1998; Yang *et al.*, 2003).

The initial decay of the control CAPS current (due to desensitization) was fitted well by a single exponential with a time constant of 39 ± 5 s. The rapid decay that took place within the first 2 min of CAPS application produced a > 70% reduction from the initial peak response and was then followed by a more slowly decaying component (Fig. 1A–C; average time constant 7.5 ± 2.1 min). The ratio of the amplitudes of rapidly and slowly decaying components of the fitted currents (see Materials and methods) was somewhat variable from cell to cell, but the decay was nearly complete at the longest periods of observation (20 min; Fig. 2A and B, dotted line).

## Activation of $NK_2$ receptors enhances CAPS currents and slows their desensitization

SP ($n$ = 6) or NKA ($n$ = 5) applied 1–2 min before CAPS enhanced the amplitude of the CAPS currents by 3.9 ± 1.2- and 4.4 ± 2.5-fold, respectively, and also reduced the lag time of CAPS responses by about 30% (4 s vs. 6.6 s in the absence of NKs; Fig. 1F). Application of the NKs before CAPS or in the initial 2–5 min after CAPS administration during the period of partial desensitization slowed the desensitization rates of CAPS currents by 3.8-fold and 3.6-fold, respectively (Figs 2 and 3A). The facilitated CAPS responses had a considerably slower time to peak and rates of desensitization (Fig. 2C and E) than currents elicited by NKs or CAPS alone (Fig. 1), and were fully inhibited by a TRPV1 antagonist (5 μM; Fig. 2B). The responses to either SP ($n$ = 5) or NKA ($n$ = 4) were reversed by a selective $NK_2$ antagonist, MEN 10,376 (5 μM; Fig. 2A and D), but unaffected by an $NK_3$-selective antagonist, SB 235,375 (5 μM, $n$ = 3; Fig. 2D).

SP ($n$ = 7) or NKA ($n$ = 7) applied up to 20 min after the start of continuous administration of CAPS enhanced the CAPS currents (two–fourfold increase; Fig. 2). The magnitude of the enhancement (measured as the peak current) depended on the interval between CAPS and SP or NKA administration (Fig. 4A). If SP or NKA was administered within 5 min after CAPS the enhancement was larger than at longer intervals when the CAPS current exhibited greater desensitization (Fig. 4B). In contrast to NKA and SP, neither an $NK_1$-selective agonist, SarMetSP (Fig. 5C and D) nor an $NK_3$-selective agonist, NKB (Fig. 5C and D) altered the CAPS responses. Taken together these data suggest that $NK_2$ receptors, but not $NK_3$ or $NK_1$ receptors, mediate the facilitatory effect of SP and NKA on CAPS responses.

## The effect of NKs on CAPS currents is mediated by PKC activation

Because PKC has been implicated in the modulation of TRPV1 desensitization rates (Koplas *et al.*, 1997; Premkumar & Ahern, 2000; Sculptoreanu *et al.*, 2005b) and is presumed to mediate some of the effects of NKs in DRG neurons (Sculptoreanu & de Groat, 2003, 2007), we evaluated the role of PKC in the NK-induced changes in TRPV1 currents. We tested the effects of agents that stimulate PKC at concentrations of CAPS (0.5 μM) that would be expected to

produce currents 20% of the maximal responses evoked at saturating concentrations. Therefore if phosphorylation simply sensitized responses to CAPS, it would be expected to cause a maximum fivefold increase in the currents. Indeed, PDBu as well as SP and NKA produced a level of facilitation of the CAPS currents consistent with a sensitization that shifted TRPV1 responsiveness to lower concentrations of CAPS. We also examined the interactions between facilitated CAPS currents and agents that inhibit PKC (BIM) or inhibit phosphoprotein phosphatases (NAcPh and DEC; Choi & Soderlund, 2004; Sculptoreanu *et al.*, 2005b).

PDBu (0.5 μM) applied prior to CAPS ($n = 6$) slowed the time to peak and slowed the desensitization of the CAPS currents, and increased the peak CAPS current amplitudes from −11 ± 2 pA/pF in untreated neurons to −34 ± 19 pA/pF in PDBu-pretreated neurons ($n = 6$; Fig. 2E). PDBu applied after partial desensitization of CAPS responses ($n = 16$) facilitated the currents by an average of nearly twofold (−21 ± 7 pA/pF for all experiments 0–20 min after CAPS). However, the enhancements were larger when PDBu was applied in the initial 10 min after CAPS (−27 ± 11 pA/pF) than when it was applied at later times (−16 ± 9 pA/pF at > 10 min; Fig. 4, filled circles). The effect of PDBu on CAPS currents was occluded by prior treatment with NKA, suggesting that activation of NK receptors maximally activated PKC (Fig. 2B and E).

The PKC inhibitor BIM (0.5 μM) administered after CAPS had no measurable effect on desensitization of currents activated by CAPS alone ($n = 6$; Fig. 3D). However, BIM accelerated the desensitization of CAPS currents that were facilitated and slowed by prior application of SP ($n = 5$) or NKA ($n = 6$; Fig. 3A) or PDBu ($n = 4$; Fig. 3B). Application of BIM before CAPS reduced the peak CAPS currents by about 20% compared with the control CAPS responses ($n = 3$; Fig. 3C). CAPS currents in BIM-pretreated cells had faster time to peak (19 ± 7 s vs. 31 ± 3 s in control neurons) and desensitized faster ($\tau_{D,fast}$: 30 ± 5 s) than control ($\tau_{D,fast}$: 41 ± 3 s) CAPS currents in absence of BIM ($P < 0.001$).

When applied alone after partial desensitization of the CAPS currents, the non-selective phosphatase inhibitors, NAcPh and DEC produced transient enhancements and slowed desensitization of CAPS currents by greater than twofold (Figs 4 and 5C, filled bars). It is noteworthy that the facilitatory effects of phosphatase inhibition were smaller (50%) than the facilitatory effects of NKs or PDBu. Thus, endogenously active PKC may maintain some basal level of TRPV1 phosphorylation in the absence of PKC activation by an exogenous agent. The CAPS currents facilitated by NKA (Fig. 2E) or PDBu (Fig. 5) were also further increased in amplitude (50–200%) by NAcPh (0.5 μM) or DEC (0.5 μM; Fig. 5). In the presence of phosphatase inhibitors (NAcPh or DEC), BIM (5 μM, $n = 5$ cells) failed to reverse the enhancement of CAPS-evoked currents by NKs or PDBu, consistent with the idea that phosphoprotein phosphatase inhibition fully prevented dephosphorylation.

Figure 4 summarizes the average response to CAPS alone (X) and after application of agents that acted to promote PKC phosphorylation (filled symbols) or to inhibit dephosphorylation of TRPV1 (open symbols). The response to CAPS alone was fitted by the sum of one exponential rising and two exponential decaying curves. It is noteworthy that the magnitude of the currents induced by agents that facilitated the CAPS currents also decayed exponentially as CAPS responses desensitized. Prior application of a phosphatase inhibitor (Fig. 5A and B) slowed the decline in response to PDBu ($n = 4$) or NKA ($n = 3$) or SP ($n = 5$) that occurred during the desensitization of CAPS currents. For example, the facilitated currents in response to PDBu applied at times > 10 min after CAPS administration had amplitudes of 16 ± 9 pA/pF ($n = 5$) in the absence of NAcPh and 25 ± 7 pA/pF ($n = 5$) when NAcPh was present prior to PDBu application (Fig. 5D).

### SP enhances CAPS-evoked increases in intracellular calcium concentration in DRG neurons

TRPV1 channels are highly permeable to $Ca^{2+}$ (Mandadi *et al.*, 2004), and changes in intracellular $Ca^{2+}$ affect phosphorylation–dephosphorylation processes (Koplas *et al.*, 1997; Mandadi *et al.*, 2004). Thus, we investigated whether TRPV1 activation leads to changes in the intracellular $Ca^{2+}$ and whether these changes are affected by SP application. Short applications (3–5 s) of CAPS (0.2–1.0 μM) increased intracellular $Ca^{2+}$ in 41.8% of DRG neurons (115/275; cell diameter 22.3 ± 0.5 μm; range 11.1–37.7 μm; average amplitude of responses was 72.2 ± 8.1 $\Delta R/R$, $n = 115$). SP (0.2 μM, 2 min application time) increased intracellular $Ca^{2+}$ in 33.3% of CAPS-responsive neurons (5/15) with an average amplitude of 5.2 ± 0.5 $\Delta R/R$. Repetitive applications of CAPS (0.2–0.5 μM, 3–5 s application time) every 6–8 min produced $Ca^{2+}$ responses that decreased over time (Fig. 6A, D and F; $n = 12$ cells; cell diameter 19.12 ± 1.2 μm; range 13.8–27.7 μm; $P < 0.05$ when compared with the first CAPS response). In a separate group of cells, SP (0.2 μM) was applied for 2 min after the third CAPS application. Regardless of whether the cells responded to SP with an increase in intracellular $Ca^{2+}$, the subsequent responses to CAPS increased (Fig 6B and G; $n = 15$ cells; cell diameter 20.3 ± 0.8 μm; range 15.4–26.2 μm; $P > 0.05$ when comparing the first CAPS response with the fourth, fifth and sixth CAPS responses). The percentage decrease of the CAPS response from the first to the fourth CAPS response was 80.06% in control conditions and 30.74% after SP treatment (unpaired *t*-test, $P < 0.05$).

## Discussion

In this study we investigated the mechanisms of desensitization of CAPS currents in response to prolonged application of CAPS and the reduction in CAPS-evoked $Ca^{2+}$-transients in response to repeated brief applications of CAPS (termed tachyphylaxis). The study revealed decreased desensitization rates and enhancement of CAPS currents in the presence of SP or a selective $NK_2$ agonist, NKA. The results also suggest that the NK-dependent changes in TRPV1 currents are related to PKC-induced phosphorylation of the TRPV1 channel (Zhou *et al.*, 2003). Although mechanistically distinct from acute desensitization, tachyphylaxis induced by repeated brief applications of CAPS seems to share some important features, such as $Ca^{2+}$-dependence and the ability of PKC to restore responsiveness and prevent the decline in CAPS responses (Koplas *et al.*, 1997; Mandadi *et al.*, 2004). Thus, it is noteworthy that SP reduced both desensitization and tachyphylaxis in our experiments. These findings raise the possibility that under acute or chronic pathological conditions, SP or NKA released from afferent nerves act on NK autoreceptors to sensitize afferent terminals in part by PKC-induced phosphorylation of TRPV1 channels.

Several features of CAPS-evoked currents were modified by activation of PKC with PDBu, or with NK agonists (SP and NKA). First the significant lag time (> 6 s) between the application of CAPS and the onset of CAPS currents was shortened by > 30% by prior treatment with PDBu. Second, PDBu applied after partial desensitization of CAPS-evoked currents enhanced the currents with a similar time course (lag time and rate of rise) to that of CAPS currents facilitated by prior exposure to PDBu. Third, the time to peak of CAPS currents, which is relatively slow (> 30 s), was significantly slowed by prior application of PDBu. Fourth, with prolonged application of CAPS, the currents exhibited an initial rapid desensitization followed by a slower desensitization. Pretreatment with PDBu or application of PDBu after partial desensitization of CAPS currents elicited currents greater in magnitude than control currents. These currents also desensitized at rates significantly slower than the currents evoked by CAPS alone (Figs 2 and 3). The enhancement of CAPS responses by NKs (SP or NKA) and slowing of desensitization were comparable to those elicited by PDBu. The effects of NKs were most likely mediated by PKC as they were readily reversed by a PKC inhibitor, BIM, and the enhancement by NKs occluded further effects of PDBu, which directly activates PKC.

Interestingly, the enhancement of CAPS currents by SP or NKA had a shorter lag time than the normal CAPS currents, but similar to that of the PDBu enhancement of partially desensitized CAPS currents. This difference in lag time suggests that multiple steps precede the opening of TRPV1 channel by CAPS and that phosphorylation by PKC accelerates these transitions.

As previously reported by Yang *et al.* (2003), we found that the non-selective NK agonist SP and NKs selective for $NK_1$, $NK_2$ and $NK_3$ receptors evoked a rapid onset and rapidly desensitizing current when applied alone. These currents must be mediated by a channel different than TRPV1 because prior application of a TRPV1 antagonist did not prevent NK-evoked inward currents (Fig. 1C). However, the rapid responses were not detected if NK agonists were applied in the presence of CAPS. Thus, when applied after partial desensitization of CAPS currents, the SP- and NKA-enhanced CAPS currents were slow, having onset time courses similar to those of the PDBu-enhanced CAPS currents. The currents also exhibited slow desensitization and were blocked by the TRPV1 antagonist. These observations suggest that activation of TRPV1 may cross-desensitize NK-activated non-selective currents elicited by all subtypes of NK receptors, whereas the NK facilitatory effects on TRPV1 responses may be mediated by only one subtype of NK receptor ($NK_2$). Consistent with this idea the effect of SP on CAPS-evoked currents was antagonized by an $NK_2$ antagonist but not an $NK_3$ antagonist. In addition, $NK_1$- and $NK_3$-selective agonists had no detectable effects on TRPV1 responses, suggesting that $NK_2$ receptors were solely responsible for modulation of TRPV1 by NKs in our experiments. A similar cross-desensitization by TRPV1 activation has been reported for $P_2X$ receptors in DRG neurons (Piper & Docherty, 2000). Recently, Zhang *et al.* (2007) reported that $NK_1$ receptors can elicit changes in TRPV1 responses similar to those reported here for $NK_2$ receptors. We believe that even though these findings seem superficially contradictory, our studies very likely focus on different subpopulations of DRG neurons that may indeed be different with respect to the subtype of NK receptor expressed. First, the neurons in the Zhang *et al.* (2007) study were on average = 20 μm, while those chosen in our study were = 30 μm and averaged > 50 pF. However, our cells were similar in size to TRPV1-positive populations of adult rat DRG neurons reported by other labs (see for example the diameter of TRPV1-immunostained neurons in fig. 3 of Liu *et al.* (2006); and type 5 and 8 neurons in Rau *et al.* (2006). Secondly, the rats used in the Zhang *et al.* (2007) study (28–35 days postnatal) were in general younger than those used in our studies, which were 200–250 g or 2–3 months old. Indeed we found that the sensitivity of CAPS currents to modulation by NKs was reduced in younger animals (unpublished studies). Thirdly, we looked at caudal lumbar and sacral ganglia, while Zhang *et al.* (2007) used rostral lumbar segments that innervate different organs and may behave differently. All of the above could account in part for differences in the results of these two studies; but the most telling difference, the difference in the size of neurons tested, most likely reflects a selection of different populations of DRG neurons.

The $Ca^{2+}$-imaging results indicate that tachyphylaxis, which was observed with repetitive CAPS applications, was diminished after SP treatment (Fig. 6). Similar reversal of tachyphylaxis was reported in other studies after activation of PKC (Mandadi *et al.*, 2004). Tachyphylaxis was shown to be proportional to the CAPS-evoked increase in intracellular $Ca^{2+}$ by Koplas *et al.* (1997) and in the present work (compare B and C in Fig. 6). Thus, prolonged elevation of intracellular $Ca^{2+}$ may be a possible mechanism for tachyphylaxis by activating $Ca^{2+}$-dependent phosphatases that dephosphorylate TRPV1 (Koplas *et al.*, 1997). Consistent with this idea, our experiments showed that intracellular $Ca^{2+}$ continued to be elevated above baseline long after CAPS application (Fig. 6A). Both desensitization and tachyphylaxis seem to depend on intracellular $Ca^{2+}$ (Koplas *et al.*, 1997), suggesting that some processes triggered by alterations in the intracellular $Ca^{2+}$, such as activation of $Ca^{2+}$-dependent phosphatases (calcineurin), may be the common pathway. Previous studies suggested that desensitization and tachyphylaxis are independent phenomena (Koplas *et al.*,

1997). However, our study indicates that they may have a common or overlapping intracellular signaling mechanism as SP affected both desensitization (Fig. 2) and tachyphylaxis (Fig. 6).

Sensitization of TRPV1 responses by PKC phosphorylation is now recognized as an important step in nociceptive sensitization, allodynia and hyperalgesia (Zhou *et al.*, 2003). TRPV1 channels are targets for phosphorylation by a number of protein kinases, including protein kinase A (PKA; Distler *et al.*, 2003; Mohapatra & Nau, 2003; Varga *et al.*, 2006), PKC (Numazaki *et al.*, 2002; Bhave *et al.*, 2003; Wang *et al*., 2004; Varga *et al.*, 2006) and CaMKII (Price *et al*., 2005) that enhance the responsiveness of TRPV1 receptors to CAPS and other activators. On the other hand, dephosphorylation by calcineurin inhibits the responsiveness to agonists and speeds up desensitization in the presence of agonist (Docherty *et al.*, 1996; Mohapatra & Nau, 2005). Our data suggest that there may be considerable basal PKC activity as inhibition of phosphatases by either a non-selective inhibitor (NAcPh) or a calcineurin inhibitor (DEC) enhanced the CAPS currents by as much as twofold (Fig. 5) and slowed desensitization rates, effects that were reversed by a PKC inhibitor, BIM. Indeed, consistent with a role of calcineurin in desensitization, Jeske *et al.* (2006) have shown recently that the cannabinoid WIN55212-2 speeds up TRPV1 desensitization by activating phosphatase 2B (calcineurin) and dephosphorylation of $Thr^{144}$ and $Thr^{370}$ amino acid residues in TRPV1 channels. These residues have been previously shown to be substrates for PKA phosphorylation (Mandadi *et al*., 2006).

The enhancement of CAPS responses by phosphatase inhibitors in our experiments was smaller than the enhancement by agents that stimulated phosphorylation. These data suggest that in DRG neurons some basal PKC activity maintains TRPV1 in a partially phosphorylated state that is controlled by dephosphorylation, and that there is a dynamic equilibrium between phosphorylated and dephosphorylated states of TRPV1 channels during sustained application of CAPS. Indeed, we have previously shown that in a painful bladder condition in cats (feline interstitial cystitis) in which DRG neurons are chronically sensitized, increased basal activity of PKC leads to non-desensitizing responses to CAPS that were no longer enhanced by a phorbol ester (Sculptoreanu *et al.*, 2005b). We speculated that enhanced TRPV1 activity could lead to increased firing of afferent nerves (Sculptoreanu *et al.*, 2005a) and also increased release of neuropeptides in the bladder wall.

When agents that promoted phosphorylation (PDBu, SP, NKA) or inhibited dephosphorylation of TRPV1 were applied within 1–2 min after CAPS, the amplitudes of the facilitated currents were very large, but the currents induced by these agents were significantly smaller at longer intervals after CAPS. The decline in facilitation paralleled the decline in currents evoked by CAPS alone. This time-dependent decline in the peak facilitated responses during the CAPS desensitization may reflect a slow conversion of activated TRPV1 channels into an inactive state that can not be reactivated by PKC phosphorylation (Fig. 5). By maintaining some level of phosphorylation in the presence of a phosphatase inhibitor, the conversion to a closed (unavailable) state is prevented. The actions of SP or NKA to enhance the phosphorylated state of TRPV1 by activating $NK_2$ autoreceptors on peripheral or central afferent terminals may be an important mechanism contributing to the algesic properties of NKs.

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## Abbreviations

| | |
|---|---|
| ATPγS | adenosine-5′-O-(3-thiotriphosphate) |

| | |
|---|---|
| BIM | bisindolylmaleimide |
| BSA | bovine serum albumin |
| CAPS | capsaicin |
| DEC | decamethrin |
| DMEM | Dulbecco's modified essential medium |
| DMSO | dimethylsulfoxide |
| DRG | dorsal root ganglia |
| HBSS | Hank's balanced salt solution |
| NAcPh | α-naphthyl acid phosphate |
| NK | neurokinin |
| NKA | [βAla$^{8}$]-neurokinin A (4–10) |
| NKB | [MePhe$^{7}$]-neurokinin B |
| PDBu | phorbol 12,13-dibutyrate |
| PKA | protein kinase A |
| PKC | protein kinase C |
| SarMetSP | [Sar$^{9}$, Met$^{11}$]-substance P |
| SP | substance P |
| TRPV1 | transient receptor potential vanilloid receptor 1 |

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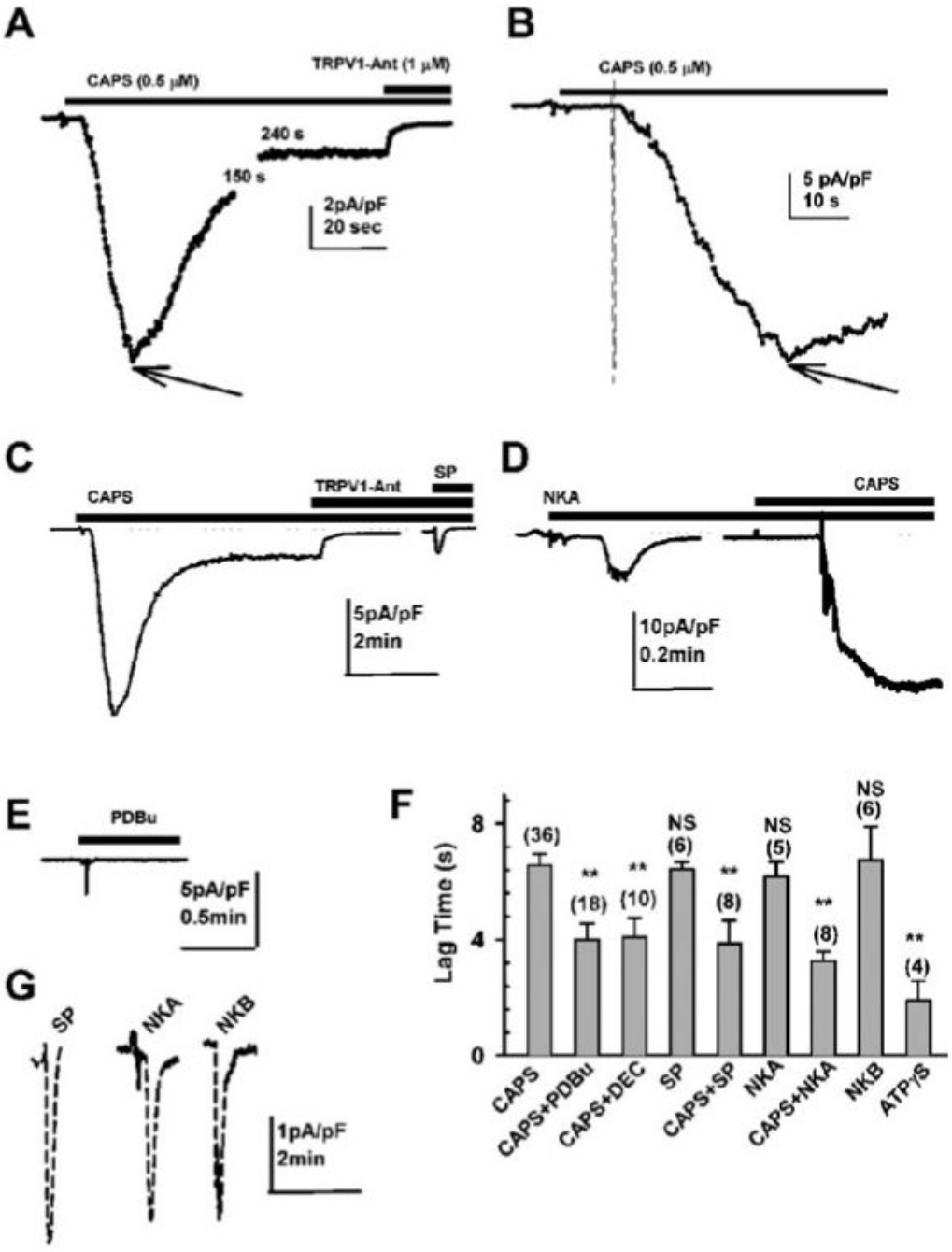


**Fig. 1.**

Time course and lag time of responses to capsaicin (CAPS), NKs [substance P (SP), [βAla$^{8}$]-neurokinin A (4–10) (NKA), [MePhe$^{7}$]-neurokinin B (NKB)] and phorbol 12,13-dibutyrate (PDBu) in DRG neurons from adult male rats. (A) Time course of response evoked by CAPS alone (0.5 μM) and block of response by a selective transient receptor potential vanilloid receptor 1 (TRPV1) antagonist (TRPV1-Ant, 1 μM). (B) Initial rise of CAPS response in (A) on an expanded scale showing the lag time of response from the time of application (solid horizontal line) to the start of the rising phase (dotted vertical line). (C) SP (0.5 μM) applied after block of CAPS response by the TRPV1 antagonist triggers a rapid onset, rapid desensitizing response. (D) NKA (0.5 μM) applied before CAPS also triggers a rapid onset, rapidly desensitizing inward

current. The lag time between NKA application and the beginning of the rising phase was similar to that of CAPS responses (5–7 s). (E) The PKC activator PDBu (0.5 μM) did not elicit a current when administered alone. Calibration bars are shown next to currents. (F) Summary of lag times of response to CAPS, SP, NKA and NKB alone or combinations of other agents. For comparison, the lag time of response to adenosine-5′-O-(3-thiotriphosphate) (ATPγS, 50 μM)-activated $P_2X$ currents is also shown. Comparison was considered statistically significant if $P < 0.05$; a one-way analysis of variance was first carried out followed by a *post hoc* comparison between the different groups using the Holm–Sidak test, as described in Materials and methods (**$P < 0.001$). (G) Inward currents in response to NKs SP, NKA and NKB. DEC, decamethrin.

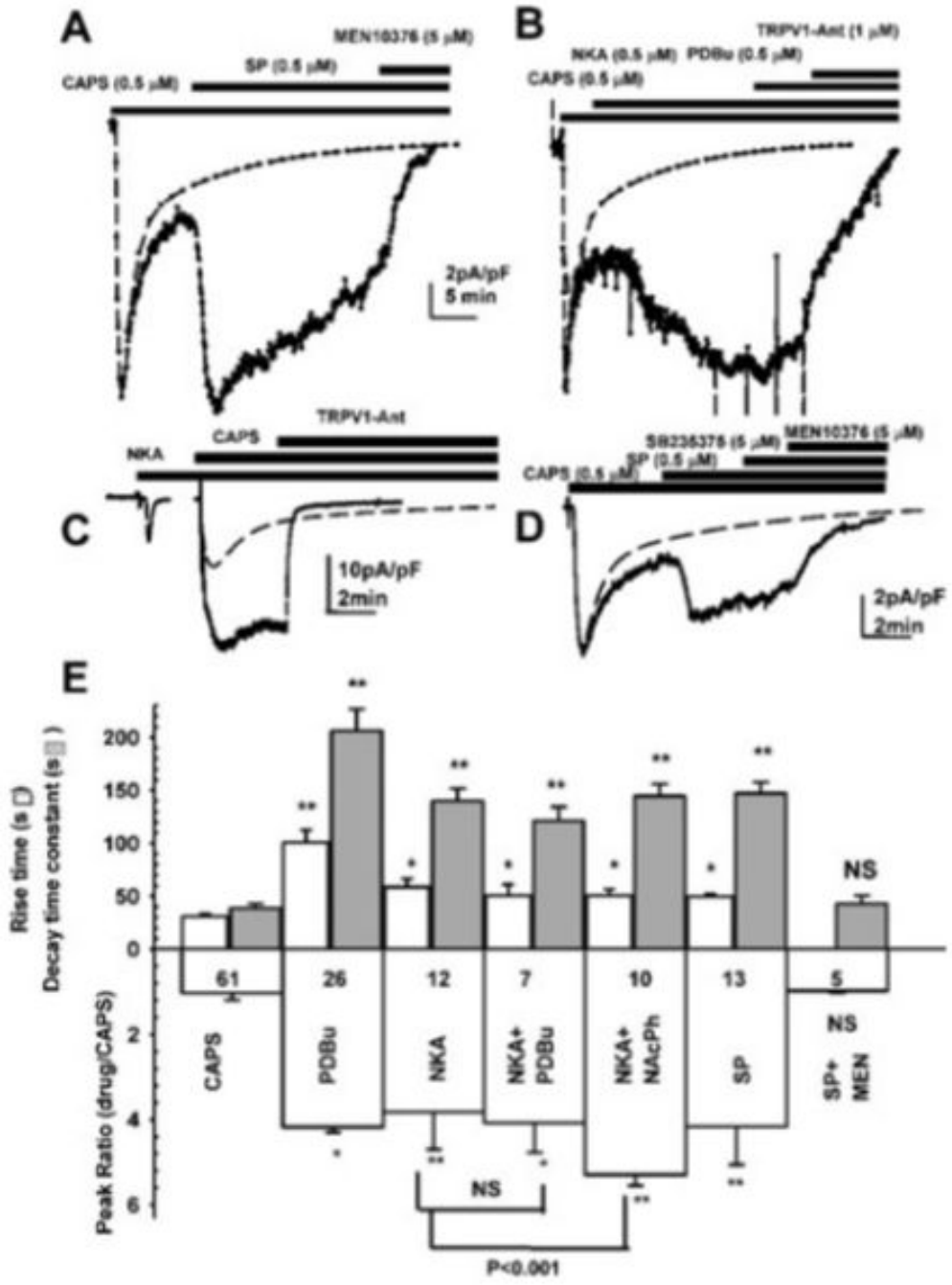


**Fig. 2.**

Effect of NKs [substance P (SP), [βAla$^8$]-neurokinin A (4–10) (NKA)] and a phorbol ester (phorbol 12,13-dibutyrate, PDBu) on capsaicin (CAPS) responses in DRG neurons from adult male rats. (A) Time course of the current induced by exposure to CAPS (0.5 μM), followed by SP (0.5 μM) and an $NK_2$-selective antagonist, MEN 10,376 (5 μM, continuous line). (B) Time course of the CAPS current induced by prolonged exposure to CAPS alone (0.5 μM, broken line) and later application of NKA (0.5 μM) followed by PDBu (0.5 μM) and a selective transient receptor potential vanilloid receptor 1 (TRPV1) antagonist (TRPV1-Ant, 1 μM). (C) Response to NKA alone (0.5 μM) and CAPS (0.5 μM) applied after full desensitization of NKA response. A selective TRPV1 antagonist (TRPV1-Ant, 1 μM) applied later fully inhibited the current. (D)

Time course of the CAPS current induced by prolonged exposure to CAPS alone (0.5 μM) and later application of SP (0.5 μM), the $NK_3$ antagonist SB 235,375 (5 μM) and MEN 10,376 (5 μM, continuous line). Drugs were applied as shown by thick bars above current traces. For comparison, the broken line in (A–D), the average fit of responses to CAPS alone in 20 neurons, is also shown. (E, top) Average time from drug application to maximum response (time to peak) and time constant of desensitization (fitted by a single exponential) after CAPS alone and after application of PDBu (0.5 μM), NKA (0.5 μM) and SP (0.5 μM) in the presence of CAPS and SP after CAPS in the presence of $NK_2$ antagonist MEN 10,376 (MEN, 0.2 M). (E, bottom) Peak currents evoked by various agents in the presence of CAPS normalized to fitted amplitudes for responses to CAPS alone at the time the effect of various agents reached a maximum. The number of experiments is shown as insets. Bar graph data are averages of pooled data, standard error of the mean (SEM) and statistical difference from control currents (two tailed *t*-test, unequal variance, $*P < 0.05$, $**P < 0.01$, NS, not significant). Comparisons in (E) were considered statistically significant if $P < 0.05$ (*); a one-way analysis of variance was first carried out, followed by *post hoc* comparisons between the different groups using the Holm–Sidak test. NAcPh, α-naphthyl acid phosphate.

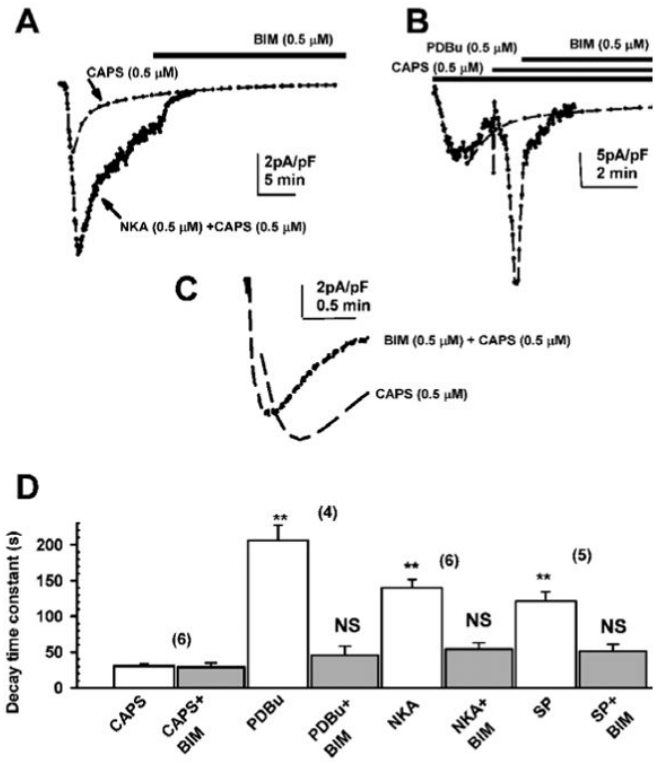


**Fig. 3.**

Effect of a PKC inhibitor on NKs [substance P (SP), [βAla$^8$]-neurokinin A (4–10) (NKA)] and a phorbol ester (phorbol 12,13-dibutyrate, PDBu) induced slowing of capsaicin (CAPS) responses in DRG neurons. (A) CAPS (0.5 μM) applied in the continuous presence of NKA (0.5 μM) followed by administration of a PKC inhibitor, bisindolylmaleimide (BIM, 0.5 μM). (B) Time course of current induced by CAPS alone (0.5 μM) and PDBu (0.5 μM) applied in the continuous presence of CAPS, after partial desensitization of the CAPS response. Dotted lines in (A) and (B) are the fit of responses to CAPS alone in 20 neurons. (C) Average traces of CAPS (0.5 μM)-evoked current densities ($n$ = 3 neurons) in which CAPS was applied at 2–3 min after application of BIM (0.5 μM) and fitted curve of average CAPS current densities in 20 neurons in response to CAPS alone. Calibration bars for A, B and C are shown. (D) Effect of BIM on currents evoked by CAPS, PDBu, NKA and SP. Average decay time constant (desensitization of the initial rapid phase fitted by a single exponential) of CAPS (0.5 μM) alone, and currents elicited by PDBu (0.5 μM), NKA (0.5 μM) and SP (0.5 μM) in the presence of CAPS (0.5 μM). The number of experiments is shown above the graph. Bar graph data are averages of pooled data, standard error of the mean (SEM) and statistical difference from control currents (two-tailed $t$-test, unequal variance, $^{**}P < 0.01$, NS, not significant). Comparisons in (D) were considered statistically significant if $P < 0.05$; a one-way analysis of variance was first carried out followed by a *post hoc* comparison between the different groups using the Holm–Sidak test.

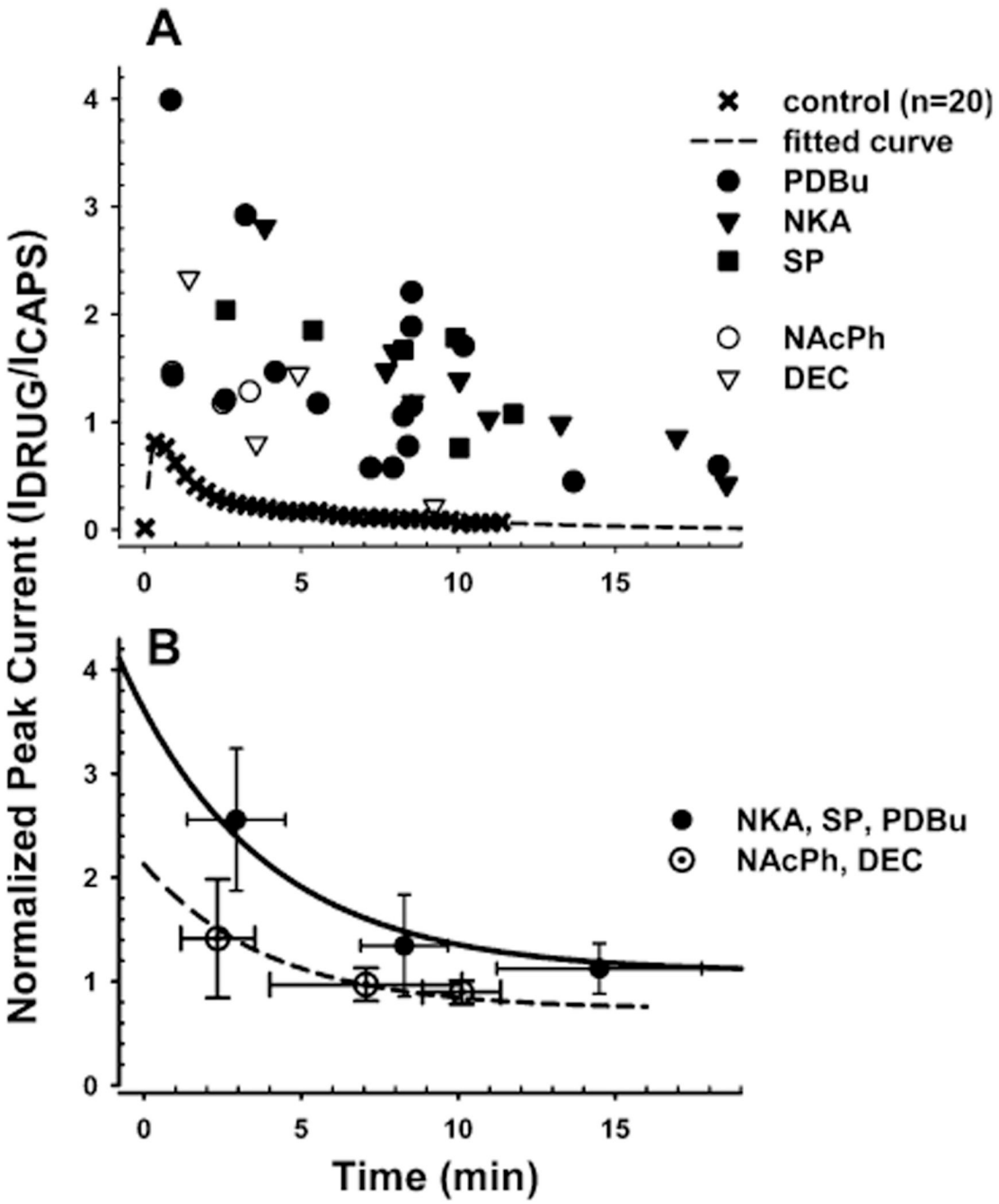


**Fig. 4.**

The enhancement of capsaicin (CAPS) currents by agents that promote phosphorylation or inhibit dephosphorylation. The peak magnitude of the currents declines with time in the continuous presence of CAPS. (A) Average response to CAPS alone (X, *n* = 20 cells) normalized to maximum amplitude and after applications of agents that are presumed to promote PKC-mediated TRPV1 phosphorylation (filled symbols) or to inhibit dephosphorylation (empty symbols). The response to CAPS alone was fitted by a sum of one exponential rising and two exponential decaying curves (dotted line, equation shown above graph, also see Materials and methods). (B) The increase of CAPS currents in response to agents that facilitated the currents. The extent of facilitation decayed exponentially as the CAPS

responses desensitize. Data in (A) for effects of various agents that either promoted phosphorylation (filled circles) or inhibited dephosphorylation (empty circles) were grouped in early, intermediary and late time segments and averaged as shown. DEC, decamethrin; NAcPh, α-naphthyl acid phosphate; NKA, [βAla$^{8}$]-neurokinin A (4–10); PDBu, phorbol 12,13-dibutyrate; SP, substance P.

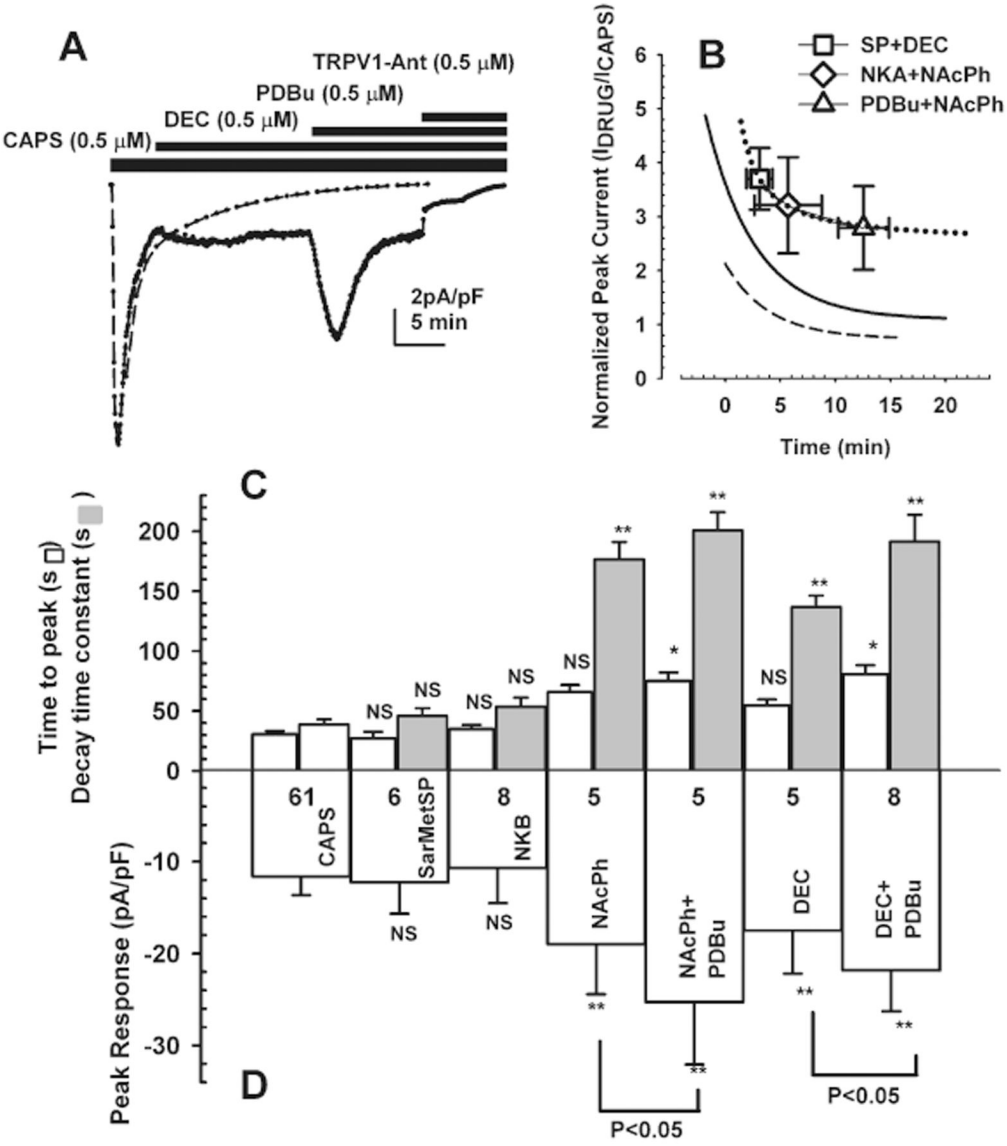


**Fig. 5.**

Effect of $NK_1$ ([$Sar^9$,$Met^{11}$]-substance P, SarMetSP) and $NK_3$ ([MePhe7]-neurokinin B, NKB) receptor agonists and phosphatase inhibitors [α-naphthyl acid phosphate (NAcPh), decamethrin (DEC)] on capsaicin (CAPS) responses in DRG neurons from adult male rats. (A) Time course of the response to CAPS (0.5 μM) and DEC (0.5 μM) applied in sequence. Phorbol 12,13-dibutyrate (PDBu; 0.5 μM) was applied at > 20 min after application of CAPS and DEC as shown by bars above the graph. A selective transient receptor potential vanilloid receptor 1 (TRPV1) antagonist (TRPV1-Ant, 0.5 μM) fully inhibited the current. The dotted line is the fit of average responses to CAPS alone in 20 neurons. (B) Prior application of phosphatase inhibitors NAcPh (0.5 μM) or DEC (0.5 μM) slowed the decline in the response to PDBu ($n$ =4) or [$\beta Ala^8$]-neurokinin A (4–10) (NKA; $n$ = 3) or substance P (SP; $n$ = 5) that occurred during the desensitization of CAPS currents (continuous line). Continuous and dotted lines are fits shown in Fig. 4B. (C) Average time to peak (rise time) and decay time constant (initial rapid phase of desensitization fitted by a single exponential) after CAPS (0.5 μM) and SarMetSP (0.5 μM), NKB (0.5 μM), NAcPh (0.5 μM) and DEC (0.5 μM) in the presence of CAPS. (D) Peak

current densities after CAPS (0.5 μM) and SarMetSP (0.5 μM), NKB (0.5 μM) NAcPh (0.5 μM) and DEC (0.5 μM) in the presence of CAPS. The number of experiments is shown as insets. Bar graph data are averages of pooled data, standard error of the mean (SEM) and statistical difference from control currents (two tailed *t*-test, unequal variance, $*P < 0.05$, $**P < 0.01$, NS, not significant). Comparisons in (C) and (D) were considered statistically significant if $P < 0.05$ (*); a one-way analysis of variance was first carried out, followed by a *post hoc* comparison between the different groups using the Holm–Sidak test.

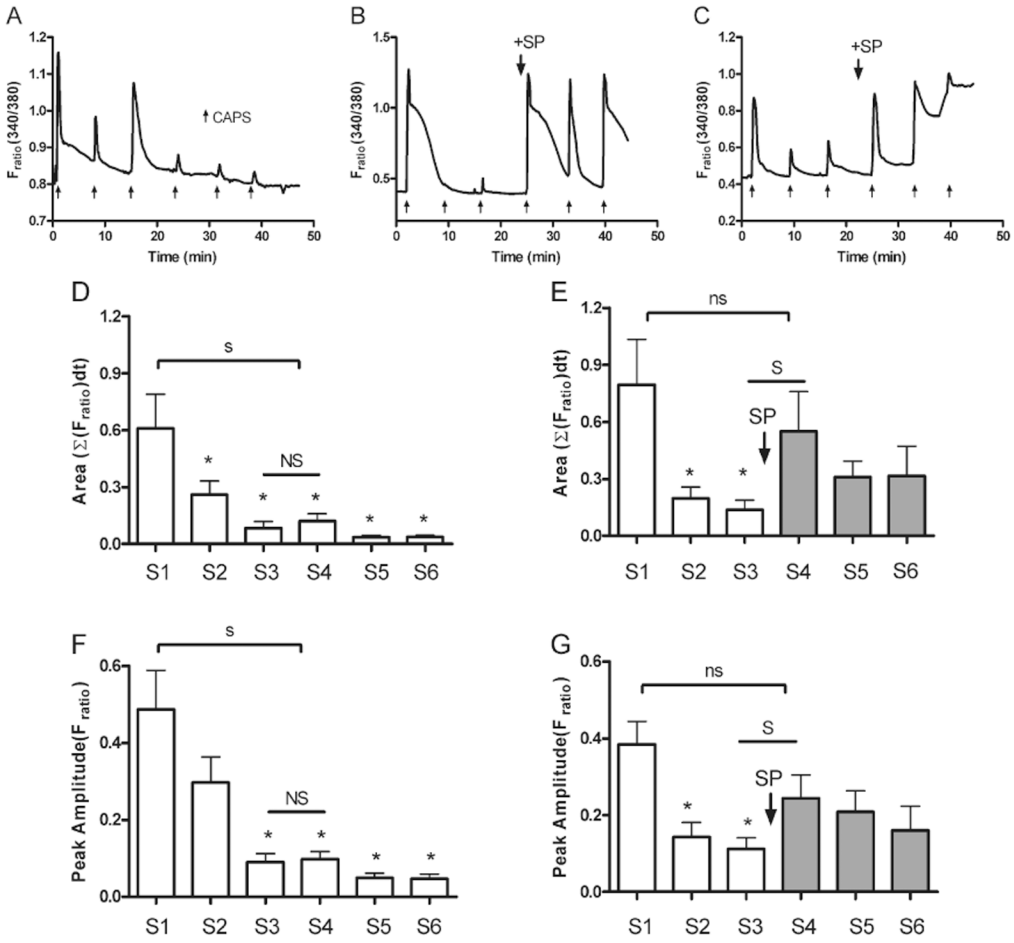


**Fig. 6.**

Effect of substance P (SP) on tachyphylaxis of capsaicin (CAPS)-induced increases in intracellular $Ca^{2+}$ in cultured DRG neurons. (A) Example from a DRG neuron responding with increases in intracellular $Ca^{2+}$ to six consecutive applications of CAPS (0.2 μM, 3–5-s duration; arrows) every 6–8 min. (B, C) Examples from two DRG neurons responding with increases in intracellular $Ca^{2+}$ to six consecutive applications of CAPS (0.2 μM, 3–5-s duration), every 6–8 min before (first three applications) and after (last three application) SP treatment (0.2 μM, 2 min). (D, F) Summary of the area under the curve (D), response amplitude (F) from cells responding to six consecutive CAPS stimuli (S1–S6; 0.2 μM, 3–5-s duration; $n = 12$ cells) in control conditions. (E, G) Summary of the area under the curve (E), response amplitude (G) from cells responding to six consecutive CAPS stimuli (0.2 μM, 3–5-s duration; $n = 15$ cells) in control conditions S1–S3 and after SP (0.2 μM, 2 min) treatment S4–S6. Statistical significance was tested using ANOVA followed by Tukey–Kramer *post hoc* test. Asterisks indicate statistical significance (*$P < 0.05$) in between S1 and S2, S3, S4, S5, S6. S indicates statistical significance ($P < 0.05$) in between S3 and S4; s indicates statistical significance ($P < 0.05$) between S1 and S4; ns, NS indicate no statistical significant differences ($P > 0.05$).