

An antinociceptive role for substance P in acid-induced chronic muscle pain

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Release of substance P (SP) from nociceptive nerve fibers and activation of its receptor neurokinin 1 (NK1) are important effectors in the transmission of pain signals. Nonetheless, the role of SP in muscle pain remains unknown. Here we show that a single i.m. acid injection in mice lacking SP signaling by deletion of the tachykinin precursor 1 (*Tac1*) gene or coadministration of NK1 receptor antagonists produces long-lasting hyperalgesia rather than the transient hyperalgesia seen in control animals. The inhibitory effect of SP was found exclusively in neurons expressing acid-sensing ion channel 3, where SP enhances M-channel-like potassium currents through the NK1 receptor in a G protein-independent but tyrosine kinase-dependent manner. Furthermore, the SP signaling could alter action potential thresholds and modulate the expression of TTX-resistant sodium currents in medium-sized muscle nociceptors. Thus, i.m. SP mediates an unconventional NK1 receptor signal pathway to inhibit acid activation in muscle nociceptors, resulting in an unexpected antinociceptive effect against chronic mechanical hyperalgesia, here induced by repeated i.m. acid injection.

dorsal root ganglion | fibromyalgia | genistein | G-protein-coupled receptor | Src

Substance P (SP) is an undecapeptide belonging to the tachykinin small-peptide family (1). SP is generated in primary nociceptive sensory neurons (nociceptors) and is released with noxious stimulation (2). The release from cutaneous peripheral terminals induces neurogenic inflammation and release from central terminals enhances the glutamate-dependent excitatory postsynaptic potential, thus leading to central sensitization (3–5). Although sensory neurons in muscle also contain SP, i.m. SP injection evokes a very low level of neurogenic inflammation and no pain (6, 7).

Accumulating evidence has suggested that muscle pain might be closely related to acidosis and activation of proton-sensing ion channels (8–10). Lactate and ATP sensitize this acid activation (11, 12). Human and animal studies have revealed that acidosis is an effective trigger of muscle pain. Thus, chronic muscle pain can be induced in rodents by repeated i.m. injection of acid (13, 14), by i.m. injection of complete Freund's adjuvant (CFA), carrageenan, capsaicin, or proinflammatory cytokines (15–18), by arterial occlusion (19), and by eccentric muscle contraction (20). These stimuli might correspond to muscle pain related to acidosis, inflammatory and ischemic myalgia, or delayed-onset muscle soreness. Although these models might not reflect fully the complicated human pain conditions, and although repetitive acidosis is not known to produce chronic pain or central sensitization in humans, these rodent models are useful for probing the underlying mechanisms and analgesic modulation of chronic muscle pain. For instance, acid-sensing ion channel 3 (ASIC3) is essential for triggering acid-induced mechanical hyperalgesia in models of i.m. injection of acid, CFA, or carrageenan (13, 17, 21–23). Coinjection of neurotrophin-3 reverses the acid-induced chronic hyperalgesia (24). Also, some muscle-derived pain can be attenuated by treatment with pregabalin or with the voltage-

dependent potassium M-channel openers retigabine and flupirtine (25, 26). Flupirtine already is in use as an analgesic for chronic muscle pain. In addition to acid, ATP, capsaicin, and nerve growth factor (NGF) also can activate muscle nociceptors (27, 28). However, the role of SP in muscle pain is unknown.

We adopted the mouse model of muscle pain induced by repeated i.m. acid injection to test the role of SP in acid-evoked hyperalgesia and then used whole-cell patch-clamp recording to probe the SP-mediated signal pathway in muscle-afferent neurons.

Results

Loss of SP Signal Facilitates Referred Hyperalgesia Induced by I.M. Acid Injection. As described by Sluka et al. (13), repeated injections of acid solution (pH 4.0) to one side of the gastrocnemius muscle (GM) caused bilateral, long-lasting (>2 wk) mechanical hyperalgesia in mouse hind paws (Fig. 1A). The first acid injection induced rapid, transient referred hyperalgesia (an increased response to a noxious stimulus outside the injured site), which declined after 24 h in wild-type mice. A second acid injection administered 5 d after the first induced long-lasting referred hyperalgesia. Mice lacking the tachykinin 1 gene (*Tac1*^{-/-}; no SP and no neurokinin A production) showed persistent referred hyperalgesia that lasted for at least 22 d (Fig. 1B and C). Therefore, the reversal of the acid-induced hyperalgesia seemed to depend on SP or neurokinin A. To evaluate the role of the SP receptor neurokinin 1 (NK1) in this process, we coinjected acid and 2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7-diphenyl-4-perhydroisoindolone (3aR, 7aR) (RP-67580), a selective NK1 antagonist, into the muscle of wild-type mice. This coinjection also induced long-lasting mechanical hyperalgesia and produced an exact phenocopy of the *Tac1* gene deletion (Fig. 1D). Therefore, SP–NK1 signaling was activated by i.m. injection of acid and counteracted the acid-induced hyperexcitation of muscle nociceptors.

SP Prevents the Development of Referred Hyperalgesia Induced by I.M. Acid Injection. We next determined whether the *Tac1*^{-/-} phenotype could be reversed by coinjection of the acid solution and an NK1-selective agonist, [Sar⁹, Met(O₂)¹¹]-SP (SM-SP) (Fig. 1E). Indeed, we observed transient hyperalgesia in *Tac1*^{-/-} mice 4 h after the coinjection, which returned to the same low level observed in wild-type mice. Thus, a single activation of the NK1 receptor completely reversed the *Tac1*^{-/-} phenotype. Strikingly, when acid and SM-SP were coinjected again in these mice, they

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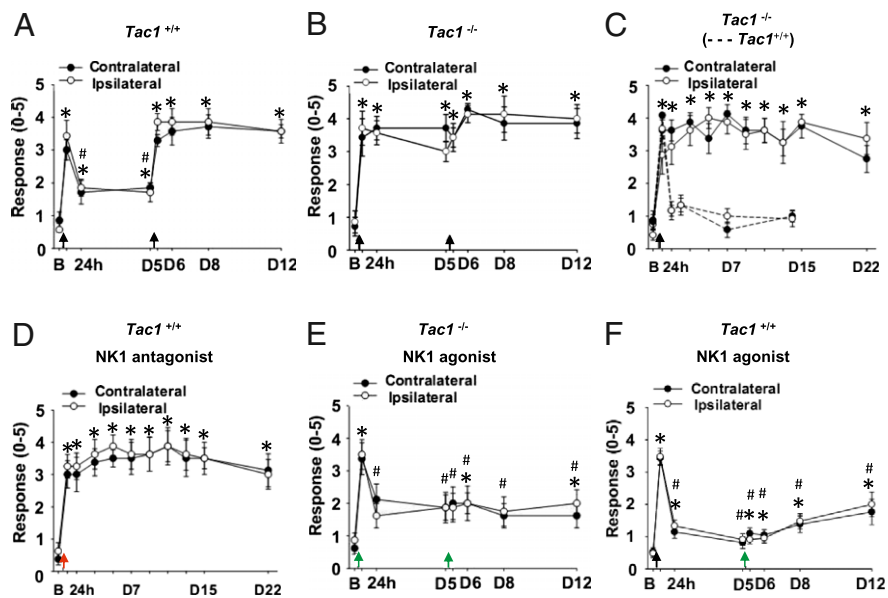


Fig. 1. Effect of SP signaling on i.m. acid-induced mechanical hyperalgesia. The withdrawal responses of mouse hind paws to a 0.2-mN bending force is shown in *Tac1*^{+/+} and *Tac1*^{-/-} mice before and after i.m. acid injection. A response was defined as foot lifting when a 0.2-mN von Frey filament was applied. (A) *Tac1*^{+/+} and (B) *Tac1*^{-/-} mice were injected with pH 4.0 saline on days 0 and 5 ($n = 7$). (C) A single i.m. acid injection evoked bilateral chronic hyperalgesia in *Tac1*^{-/-} mice ($n = 8$) but only transient hyperalgesia in *Tac1*^{+/+} mice ($n = 6$, dotted lines). (D) Coinjection of acid with 100 μ M NK1 antagonist (RP-67580) in *Tac1*^{+/+} mice induced long-lasting mechanical hyperalgesia similar to that in *Tac1*^{-/-} mice receiving a single acid injection on day 0 ($n = 8$). (E) Coinjection of acid and the 40 μ M NK1 receptor agonist SM-SP prevented i.m. acid-induced chronic referred hyperalgesia in *Tac1*^{-/-} mice; only transient hyperalgesia developed 4 h after the first coinjection ($n = 8$). (F) Coinjection of acid and 40 μ M SM-SP prevented the chronic hyperalgesia induced by the second acid injection in *Tac1*^{+/+} mice ($n = 21$). Black arrows indicate the time of i.m. acid injection. Red and green arrows indicate the time at which mice received the coinjection of acid with the NK1 antagonist and agonist, respectively. B, baseline on day 0; D, day. * $P < 0.05$ compared with baseline; # $P < 0.05$ compared with the response at 4 h.

did not show the long-lasting hyperalgesia that we previously observed. Thus, the activation of NK1 receptor signaling protects against acid-induced chronic hyperalgesia. We also could prevent the chronic hyperalgesia induced by the second acid injection by coinjection of acid and SM-SP (40 μ M) in wild-type mice (Fig. 1F). The antinociceptive effect of SM-SP at the second acid injection was dose dependent. SM-SP was as effective at 10 μ M as at 40 μ M, but a lower concentration, 4 μ M, induced a transient hyperalgesia that lasted at least 24 h after the second acid injection (Fig. S1 A–C). These data suggest that muscle nociceptors release SP in response to i.m. acid stimulation. This SP release was sufficient to reduce the hypersensitivity after the first injection but not after repeat acid administration.

Because referred mechanical hyperalgesia induced by repeated i.m. acid treatment requires ASIC3 as a transducer (13), we next examined the effect of SP deficiency in the absence of ASIC3. As expected, mice lacking *Asic3* showed no response to i.m. acid injections (Fig. S1D). Similarly, i.m. acid injection did not induce hyperalgesia in *Asic3* and *Tac1* double-knockout mice (Fig. S1E), so the antinociceptive effect of SP in muscle is observed only with ASIC3-mediated acid-induced hyperalgesia.

SP Attenuates ASIC3-Mediated Current by a G Protein-Independent Pathway in Muscle-Afferent Dorsal Root Ganglion Neurons. To determine the i.m. sites of SP action, we first investigated SP and ASIC3 immunoreactivity in muscle nerve fibers. As shown in Fig. S2A, most SP-positive nerve fibers also expressed ASIC3 channels. Acids may trigger SP release from these fibers by activating ASIC3. However, compared with saline injection, neither acid (pH 4.0) nor SM-SP injection evoked significant plasma extravasation in muscle (Fig. S2B). In contrast, injection of mustard oil into muscle significantly increased the level of plasma extravasation (Fig. S2B), indicating that muscle nociceptors can induce neurogenic inflammation that might involve a non-SP pathway.

We next used whole-cell patch-clamp recording to examine whether SP could modulate ASIC3-mediated depolarization in muscle-afferent neurons. With voltage-clamp mode, we determined ASIC3-expressing dorsal root ganglion (DRG) neurons, while the acid-induced current was inhibited by salicylic acid (SA) (29, 30). We thus named these neurons SA-sensitive (SAS) neurons (Fig. 2A). In contrast, other acid-sensitive DRG neurons expressed an SA-resistant sustained current in response to acid stimulation. Interestingly, SP specifically attenuated the acid-induced inward current in GM SAS neurons but had mixed effects on non-GM SAS neurons (Fig. 2A). Of the eight non-GM SAS neurons we recorded, two had acid-induced current positively modulated by SP (1.25 ± 0.08 ; normalized to baseline), and two were negatively modulated (0.60 ± 0.13 ; normalized to baseline); the other four neurons were SP insensitive. The cell-type-specific SP effect required NK1 receptors, because SP had no effect on ASIC3-mediated current when RP-67580 was applied (Fig. 2B). Because the NK1 receptor is a G protein-coupled receptor (GPCR), to examine the effect of G proteins, we replaced GTP with a nonhydrolysable analog, GDP- β -S in the internal pipette solution. Surprisingly, in GM SAS neurons, the effect of SP on ASIC3-mediated current was resistant to GDP- β -S dialysis, indicating the involvement of an NK1 receptor-dependent, G protein-independent pathway (Fig. 2C). In contrast, GDP- β -S dialysis significantly inhibited a baclofen-induced GABA-B current (Fig. S3). We then examined the effect of phosphotyrosine kinase (PTK) on the SP-mediated inhibition; PTK is involved in a G protein-independent pathway (31). Bath application of a PTK inhibitor, genistein, effectively abolished the SP-mediated inhibition of the ASIC3-selective current (Fig. 2D), suggesting an unconventional G protein-independent SP–NK1 signaling pathway in GM SAS DRG neurons.

SP Induces an Outward Current in Most Muscle-Afferent DRG Neurons. To probe further the mechanism of SP-mediated antinociception,

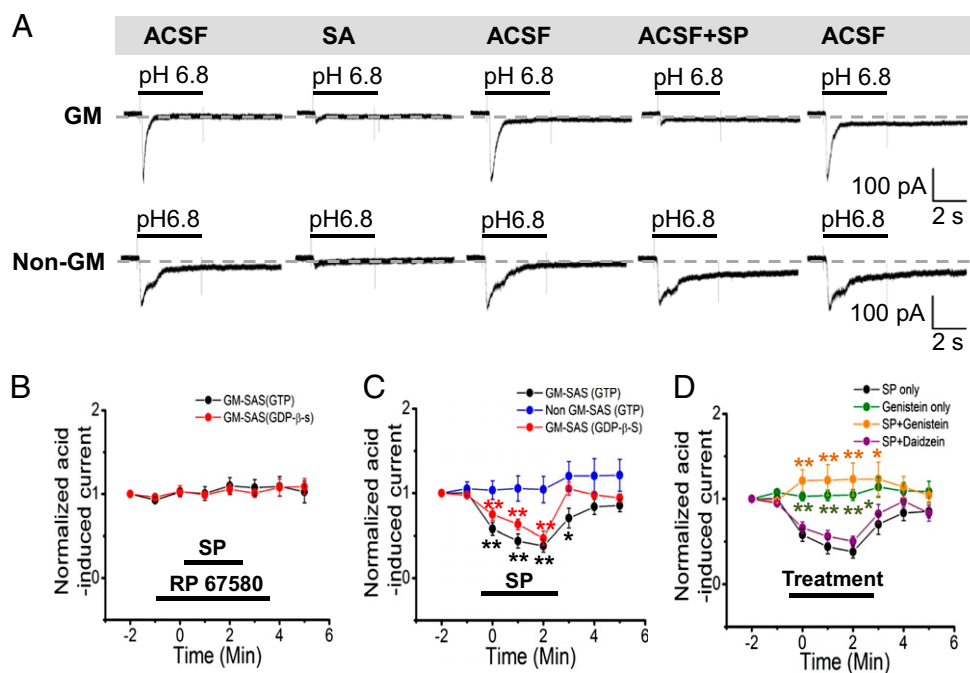


Fig. 2. Effect of SP on ASIC3-mediated currents. (A) Representative current traces showing the effect of SP on acid-induced currents in SAS neurons that innervate into GM and in non-GM SAS neurons. The pH 6.8 acid-induced currents on fluorogold-labeled GM and nonlabeled DRG neurons were screened for the presence of ASIC3 with 500 μ M SA in the bath; then the perfusion medium was switched to a bath containing 3 μ M SP to record any change in acid-induced current. At the end of the recording, the same neurons were treated with 3 μ M SP administered by puff to determine the effect of SP treatment alone. (B) GM SAS neurons were treated with the NK1 receptor antagonist RP-67580 to test whether SP-mediated modulation of acid-induced current depended on the NK1 receptor. The neurons were treated with ($n = 6$) or without ($n = 5$) a GDP- β -S-containing pipette solution to validate the participation of G proteins in SP-mediated modulation. The acid-induced currents were recorded in ACSF for 2 min as baseline, in a bath containing RP-67580 for 2 min, in a bath containing SP and RP-67580 for another 2 min, and then in a bath containing RP-67580 for 1 min. During the final minutes, neurons were treated with ACSF for washout. (C) Effect of G proteins on SP-mediated inhibition of acid-induced current. SAS neurons were treated with ($n = 9$ for GM neurons) or without ($n = 8$ for GM neurons, $n = 6$ for non-GM neurons) a pipette solution containing GDP- β -S. Acid-induced currents were recorded before (-2 min), during (3 min), and after (3 min) SP treatment. $*P < 0.05$ and $**P < 0.01$, GM SAS (GTP) vs. non-GM SAS (GTP) groups; $##P < 0.01$, GM SAS (GDP) vs. non-GM SAS (GTP) groups. (D) The involvement of PTKs in SP-mediated modulation of acid-induced current. GM SAS neurons were recorded for 2 min as baseline and then were treated with SP alone ($n = 8$) as a positive control, with genistein alone (an inhibitor of PTK; $n = 6$) as a negative control, with SP + genistein ($n = 6$), or with SP + daidzein (an inactive structural analog of genistein; $n = 9$) for 3 min. Then recorded neurons were washed out with ACSF for another 3 min. $*P < 0.05$; $**P < 0.01$ vs. SP alone.

we investigated the SP-mediated electrophysiological response in muscle nociceptors. SP (3 μ M) induced a transient inward current in 6.7% of 210 fluorogold-labeled GM-afferent neurons or a slow inactivating outward current (18.4 ± 0.9 s before returning to the baseline) in 50.5% of the neurons; the other 42.9% of the neurons did not respond to SP (Fig. 3A). In contrast, SP induced a transient inward current in 30.6% of 124 fluorogold-negative neurons and a slow inactivating outward current in 16.1%; the other 53.2% of the neurons did not respond to SP. Therefore, SP mediated an outward current (I_{SP-O}) in most muscle-afferent DRG neurons. The GM DRG neurons expressing I_{SP-O} were in a wide range of cell sizes; most were 30–40 μ m in diameter (Fig. S4), and most (9 of 14) were ASIC3-expressing neurons (Fig. S5).

The I_{SP-O} was elicited in a concentration-dependent manner (0.1–10 μ M), with an EC_{50} of 2.6 μ M ($n = 27$), and did not show a substantial decrease after repeated SP application (Fig. 3B and C). In contrast, the SP-induced transient inward current was desensitized after SP administration (Fig. 3D). While 3 μ M SP was superfused, the I_{SP-O} was reversibly blocked by the NK1-selective antagonist RP-67580 (10 μ M), with a mean inhibition of $86.0 \pm 7.6\%$ (Fig. 3E and F). In contrast, selective antagonists for the NK2 receptor [5-fluoro-3-(2-(4-methoxy-4-((R)-phenylsulphonyl)methyl)-1-piperidinyl)ethyl)-1H-indole (GR-159897); 3 μ M] and NK3 receptor [N-(α -(methoxycarbonyl)benzyl)-2-phenylquinoline-4-carboxamide (SB-218795); 3 μ M] only partly

inhibited the I_{SP-O} , with mean inhibitions of $34.4 \pm 4.1\%$ and $34.4 \pm 4.2\%$, respectively (Fig. 3E, G, and H).

Involvement of PTK Signal Pathway in I_{SP-O} . Similar to the effect of SP on ASIC3-mediated current, the I_{SP-O} was not altered after neurons were dialyzed with GDP- β -S for 10 min (Fig. 4A). To elucidate the downstream effectors of the NK1 receptor that mediated the I_{SP-O} , we examined the participation of PTK by treating neurons with genistein. Genistein significantly reduced the I_{SP-O} (Fig. 4B). Although genistein is a potent PTK inhibitor and is the most frequently used PTK inhibitor, its specificity has raised concerns, because both PTK-dependent and -independent effects have been found (32, 33). We verified the PTK dependency of I_{SP-O} on GM DRG neurons by using an inactive structural analog of genistein, daidzein, which had no effect on I_{SP-O} (Fig. 4C). The PTK-dependent effect involved Src tyrosine kinase, because 1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP1), an Src tyrosine kinase inhibitor, reduced the amplitude of I_{SP-O} (Fig. 4D and E). Moreover, if I_{SP-O} were generated in a PTK-dependent manner, application of sodium orthovanadate, a protein phosphatase inhibitor, should enhance the magnitude of I_{SP-O} . Indeed, the amplitude of I_{SP-O} increased significantly when vanadate was added to the bath solution, compared with the normal response in a bath of artificial cerebrospinal fluid (ACSF) (Fig. 5A). Vanadate also partially reversed the inhibitory effect of genistein

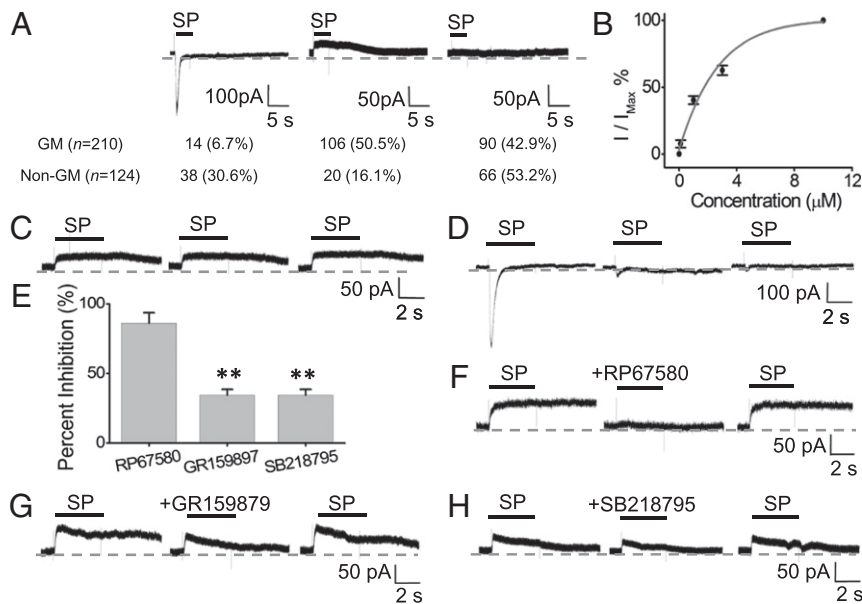


Fig. 3. SP-mediated currents in muscle DRG neurons. (A) SP-induced electrophysiological responses resulting from superfusion of 3 μ M SP for 4 s in GM-afferent DRG neurons (fluorogold positive) and non-GM-afferent DRG neurons (fluorogold negative). The number of neurons and percentage for each type of response are shown. (B) Peak whole-cell current amplitude as a function of SP (I_{SP-O}). Dose-dependent (0.1, 1, 3, and 10 μ M) response of I_{SP-O} with an EC_{50} of 2.6 μ M is shown ($n = 27$). (C) Representative traces of sustained outward current induced by repeated SP administration (I_{SP-O} , with >10 pA cutoff). (D) Representative traces of transient inward current induced by repeat SP administration (I_{SP-I} , with >10 pA cutoff). (E) Comparison of the participation of each tachykinin receptor subtype in the formation of I_{SP-O} by their respective receptor antagonists (10 μ M RP-67580 for NK1, 3 μ M GR-159897 for NK2, and 3 μ M SB-218795 for NK3). ** $P < 0.01$ vs. NK1 antagonist. (F–H) Representative traces showing the effect of the NK1 (F), NK2 (G), and NK3 (H) receptor antagonist on I_{SP-O} . All neurons were held at -70 mV for recording.

(Fig. 5B). These results demonstrate the participation of PTK in generating I_{SP-O} (Fig. 5C).

Involvement of M-Channel-Like Activity in SP-Mediated Antinociception. We next probed the ion channel mediating the I_{SP-O} . In the current-clamp mode, SP (3 μ M) induced hyperpolarization in most GM DRG neurons (38 of 49), suggesting that potassium ions may be the major charge carriers (Fig. 6A). To test this hypothesis further, we measured the SP response in ACSF con-

taining two different potassium concentrations in voltage-clamp mode. In neurons expressing I_{SP-O} , the SP response was switched to a sustained inward current when the potassium concentration was increased from 5 to 10 mM (Fig. 6B and Fig. S6A). We thus tested whether the I_{SP-O} in GM DRG neurons was mediated by the opening of potassium channels. The I_{SP-O} was abolished with tetraethylammonium chloride (TEA-Cl) treatment and reappeared again when the bath solution was switched back to normal ACSF (Fig. 6C and Fig. S6B). We then tested the possibility

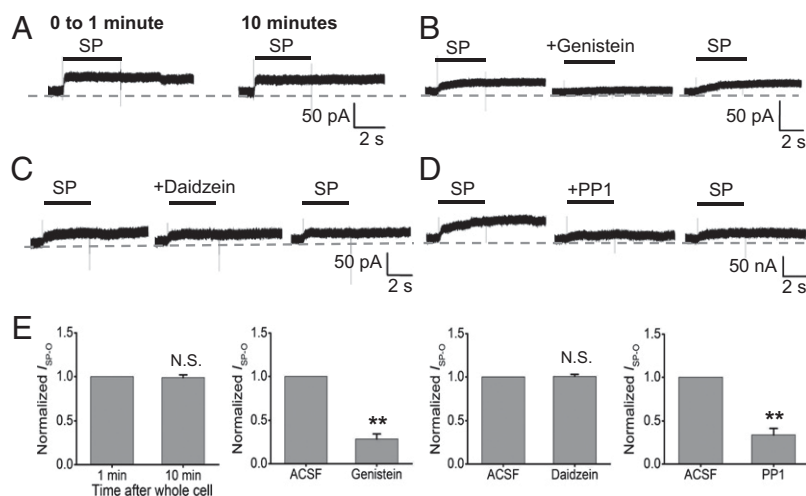


Fig. 4. Effect of PTK on I_{SP-O} . (A) Effect of GDP- β -S dialysis on I_{SP-O} . Representative traces of I_{SP-O} at baseline (0–1 min) and after 10 min of GDP- β -S (1 mM in internal solution) dialysis in the same GM DRG neuron. The dialysis did not alter the peak amplitude of I_{SP-O} significantly ($n = 17$). (B) Inhibition of I_{SP-O} by PTK inhibitor ($n = 13$). Traces represent the I_{SP-O} from the same neuron before and during superfusion with 30 μ M genistein. (C) Effect of an inactive structural analog of genistein (30 μ M daidzein) on I_{SP-O} ($n = 14$). (D) Effect of the Src-selective antagonist on I_{SP-O} ($n = 12$). Traces represent the I_{SP-O} from the same neuron before and during superfusion with 20 μ M PP1. (E) Normalized I_{SP-O} currents under different treatments. Data represent mean \pm SEM, ** $P < 0.01$, during vs. before treatment. N.S., not significant.

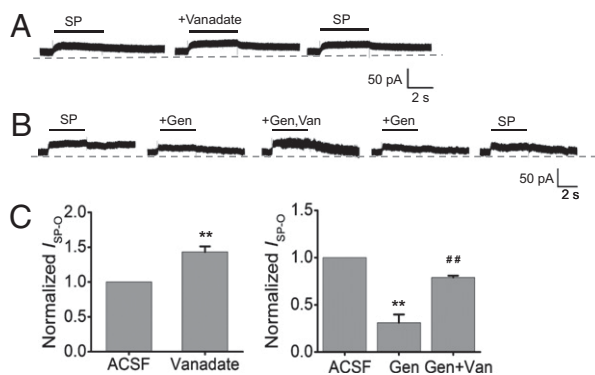


Fig. 5. Effect of protein phosphatase inhibitor on I_{SP-O} . (A) Effect of 1 μ M sodium orthovanadate on I_{SP-O} ($n = 12$). (B) The inhibitory effect of genistein (Gen) on I_{SP-O} was reversed in the presence of sodium orthovanadate ($n = 12$). Van, vanadate. (C) Normalized I_{SP-O} currents. Peak amplitude of I_{SP-O} obtained during the treatment normalized to the peak amplitude before treatment in the same neurons. Data are mean \pm SEM; ** $P < 0.01$ during vs. before treatment; ## $P < 0.01$ second vs. first treatment.

of SP opening M channels in muscle DRG neurons. In 21 of 24 GM DRG neurons, a potent M-channel blocker, 10,10-bis(4-Pyridinylmethyl)-9(10H)-anthracenone dihydrochloride (XE991) significantly inhibited the I_{SP-O} , suggesting that M channels might be the downstream effectors activated by NK1 receptor and PTK signaling (Fig. 6D and Fig. S6C). Another M-channel blocker, linopiridine, significantly inhibited I_{SP-O} , further supporting the involvement of the M channel (Fig. 6E, and Fig. S6D). We next examined whether SP could modulate M-channel activity directly. In all GM DRG neurons expressing I_{SP-O} ($n = 15$), SP showed increased M-channel activity (Fig. 6F–H and Fig. S6E). We then tested the effect of blocking M-channel activity on acid-

induced mechanical hyperalgesia in mice. Coinjection of acid with XE991 (200 μ M) into the mouse GM produced bilateral hyperalgesia at 4 h, as seen on von Frey assay, that lasted for more than 1 wk (Fig. 6I and J). Therefore, blocking M-channel activity in muscle-afferent DRG neurons produced a hyperalgesic effect on the acid-induced chronic muscle pain similar to the effect of NK1-selective antagonism.

Modulation of Neuronal Excitability in Acid-Induced Muscle Pain.

Because SP mediated the activation of M-channel activity, we further examined whether SP could alter the neural excitability in current-clamp mode in GM DRG neurons. In about half the GM DRG neurons (15/29), SP increased the action potential (AP) threshold by 6.0 ± 0.7 mV (Fig. 7A). In a small proportion of GM DRG neurons (6/29), SP also lowered AP threshold slightly, by 4.3 ± 0.4 mV. We next examined whether modulation of M-channel activity could alter neuronal excitability, similar to the effect of SP in the acid-induced muscle pain model. We compared the voltage-gated sodium currents (I_{Nav}) in GM DRG neurons 2 d after i.m. injection of acid (pH 4.0 saline) or pH 7.4 saline with or without RP-67580 (100 μ M) or XE991 (200 μ M) (Fig. 7B and C). All recordings involved fluorogold-positive small (20- to 30- μ m diameter) and medium (30- to 40- μ m diameter) GM DRG neurons. Medium-sized GM DRG neurons showed a significant alteration in voltage-gated sodium channel (Nav) excitability under different treatments. Compared with pH 7.4 saline injection, medium-sized GM DRG neurons showed significantly larger total I_{Nav} and TTX-resistant I_{Nav} after single ($n = 23$) or double ($n = 29$) injection of pH 4.0 saline (Fig. 7D). Compared with acid injection alone, medium-sized GM DRG neurons showed further enhanced total I_{Nav} and TTX-resistant I_{Nav} after coinjection of acid and RP-67580 (Fig. 7E). In contrast, acid injection had little effect on Nav excitability in small-sized GM DRG neurons. Compared with pH 7.4 saline injection, small

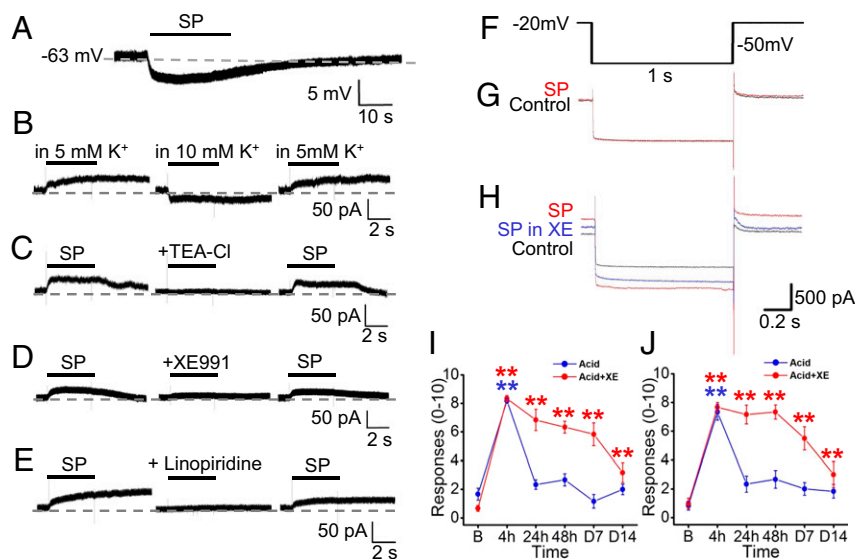


Fig. 6. Involvement of potassium-channel activity in I_{SP-O} and pain behaviors. (A) SP (3 μ M) induced hyperpolarization in most GM DRG neurons in current-clamp mode ($n = 38$ of 49). (B–E) Additional experiments to verify the participation of K^+ channel in the formation of I_{SP-O} ; neurons were held at -70 mV. (B) Effect of altering extracellular potassium concentration on SP-mediated current ($n = 15$). (C) Effect of TEA on I_{SP-O} . TEA-Cl (130 mM) replaced NaCl in the bath solution ($n = 7$). (D) Effect of the M-channel blocker XE991 (10 μ M) on I_{SP-O} ($n = 21$). (E) Effect of M-channel blocker linopiridine dihydrochloride (30 μ M) on I_{SP-O} ($n = 10$). (F) Voltage protocol used for recording M currents with and without SP. Neurons were held at -20 mV and were given a 1-s hyperpolarizing pulse to -50 mV. (G and H) Neurons were held at -70 mV to test the presence of I_{SP-O} before recording M currents. (G) SP had no effect on M currents in GM DRG neurons without I_{SP-O} ($n = 7$). (H) GM DRG neurons with I_{SP-O} showed enhancement of M currents with 3 μ M SP ($n = 15$). The enhanced effect of SP on M currents could be inhibited by 10 μ M XE991 ($n = 15$). (I) Effect of coinjection of acid and XE991 on acid-induced mechanical hyperalgesia in the ipsilateral mouse hind paw ($n = 6$ in both groups). (J) Effect of the coinjection of acid and XE991 on acid-induced mechanical hyperalgesia in the contralateral hind paw ($n = 6$ in both groups). B, baseline; D, day. Data are mean \pm SEM; ** $P < 0.01$ vs. baseline. Fig. S6 shows the statistical analyses of B–E, G, and H.

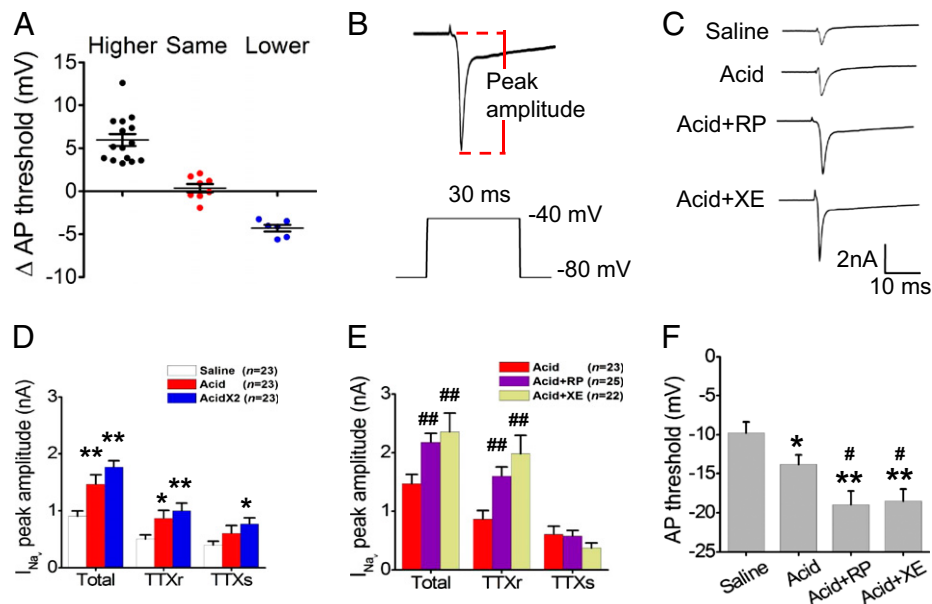


Fig. 7. Effect of SP signaling on I_{Nav} and APs in the acid-induced muscle pain model. (A) Effect of SP (3 μ M) on AP threshold in GM DRG neurons ($n = 29$). GM DRG neurons were injected with depolarizing currents of various magnitudes from resting membrane potential until an AP was formed. The AP threshold was determined by measuring the point of sharp upward rise of the AP. The delta AP threshold was calculated by subtracting the value obtained from each neuron with SP from the value without SP and was assigned to a group based on the relative change in AP threshold (higher, the same, or lower). (B) Representative current trace shows TTX-resistant I_{Nav} peak amplitude. (C) Representative TTX-resistant I_{Nav} traces in different experimental groups. (D) Effect of i.m. acid injections on I_{Nav} in medium-sized GM DRG neurons. Two days before DRG isolation, mice were injected with 20 μ L pH 7.4 saline (Saline), or with pH 4.0 saline (Acid), or with two injections of pH 4.0 saline 2 d apart (Acid \times 2). Neurons were recorded in ACSF to obtain total I_{Nav} and then were recorded in ACSF containing TTX to obtain the TTXr I_{Nav} . The value for TTX-sensitive (TTXs) I_{Nav} was calculated by subtracting TTX-resistant (TTXr) I_{Nav} from total I_{Nav} . (E) I_{Nav} in medium-sized GM DRG neurons isolated from mice that received coinjection of pH 4.0 saline and RP-67580 (100 μ M) or XE991 (200 μ M) 2 d before. (F) The AP threshold of GM DRG neurons from mice preinjected with pH 7.4 saline ($n = 29$), with pH 4.0 saline ($n = 26$), with pH 4.0 saline and RP-67580 ($n = 26$), or with pH 4.0 saline and XE991 ($n = 26$). Data are mean \pm SEM; * $P < 0.05$ and ** $P < 0.01$ vs. the saline group (D and F); # $P < 0.05$ and ## $P < 0.01$ vs. the acid group (E and F). RP, RP-67580; XE, XE991.

neurons showed significantly enhanced TTX-resistant I_{Nav} with pH 4.0 saline injection (Fig. S7A). The effect was not enhanced with coinjection of RP-67580 (Fig. S7B). Interestingly, coinjection of XE991 and acid produced a similar potentiation in terms of amplitude of I_{Nav} and specificity of the neuronal cell population, indicating that inhibition of M-channel activity and the NK1 receptor in muscle afferents produced a similar modulation of neuronal excitability specific to medium-sized GM DRG neurons (Fig. 7E and Fig. S7B). To elucidate further the effect of M-channel modulation on neural excitability, we investigated the AP threshold of medium-sized GM DRG neurons in current-clamp mode. Compared with pH 7.4 saline preinjection, medium-sized GM DRG neurons showed significantly decreased AP thresholds with pH 4.0 saline preinjection, and preinjection of acid and RP-67580 or XE991 further decreased the AP threshold (Fig. 7F). Thus, SP may modulate the opening of M channels and consequently the neuronal excitability, especially of TTX-resistant sodium currents, in medium-sized GM DRG neurons.

Discussion

The role of SP in the transmission of cutaneous nociceptive stimuli has been well studied (34–36). Substances that inhibit SP signaling pathways generally show antinociceptive effects in animal models (37–40), although NK1 antagonists failed to show an effect in most clinical trials (41). Here we uncovered an unexpected antinociceptive role for SP involving an unconventional NK1 signal pathway in muscle-afferent DRG neurons. Intramuscular release of SP seems to have an important physiological role in nociceptive plasticity by limiting the acid-induced activation of muscle nociceptors leading to referred and mirror-image hyperalgesia to a transient effect (most probably no direct effect of local SP on

central sensitization). This physiological mechanism may be therapeutically useful, because application of a selective NK1 agonist prevents long-lasting hyperalgesia after repeated acid-injection induction. The antinociceptive effect of SP seems to be mediated by ASIC3-expressing muscle-afferent neurons by attenuating the acid-induced depolarization. Our results suggest a model in which the activation of acid-sensitive muscle nociceptors triggers the local release of SP, which may act by an autocrine mechanism or specifically on ASIC3-expressing neurons, to attenuate the acid-induced depolarization by triggering M-channel-like activity. This effect involves an NK1 receptor- and a G protein-independent but PTK-dependent pathway (Fig. 8). This mechanism is not sufficient to prevent the hypersensitivity of muscle nociceptors after repeated acid stimulation.

SP signaling in muscle-afferent neurons is unique in its inhibitory transduction via a G protein-independent pathway, in contrast to the usually excitatory effects of SP signaling in almost all neuronal cells, including neurons in the spinal dorsal horn, brainstem A7 cell group, hippocampus, ventral tegmental area, and DRG (5, 31, 42, 43). One exception is SP mediating an outward current in the vagal sensory neurons in ferrets (44). However, no other study has focused on this phenomenon. Therefore, whether the vagal sensory neurons have a biological effect similar to that of muscle-afferent neurons in terms of SP signaling is an unknown but an intriguing possibility. SP signaling through the NK1 receptor is mediated by various G protein-dependent second-messenger systems, including phospholipase C, phospholipase A2, and adenylate cyclase (45). Recently SP was found to open a cation channel complex, sodium leak channel nonselective (NALCN), which is responsible for background Na^+ leak conductance, via the activation of Src family kinases without the aid of G proteins (31). The I_{SP} generated by

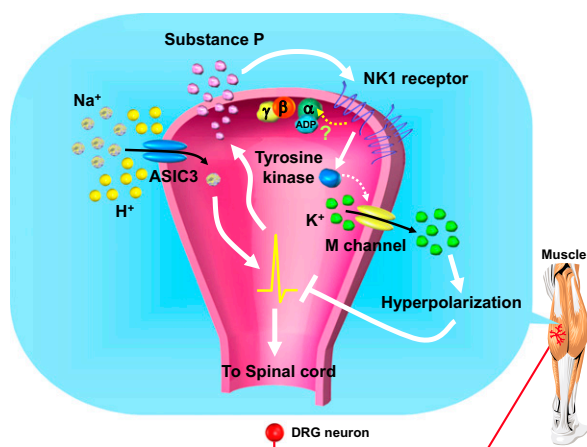


Fig. 8. A schematic model of SP-mediated inhibition of ASIC3 signaling. When tissue acidosis occurs in muscle, protons depolarize the muscle afferents by activating ASIC3 channels and fire APs, which can trigger the release of SP in the nerve terminals. SP acts on the NK1 receptor in the local nerve terminals. The NK1 receptors on muscle afferents are coupled with an unconventional signal pathway by activating tyrosine kinase and M channels to inhibit the ASIC3-mediated neural firing.

NALCN shares similarities with the I_{SP-O} we identified. None of these SP-induced currents required the direct participation of G proteins, but both depended on PTK. That SP antinociception in muscle acts via an Src kinase is intriguing because Src kinases can be activated downstream of the neurotrophin receptor (46, 47). Given that i.m. administration of neurotrophin 3 reverses the acid-induced hyperalgesia, SP may work through or in parallel with neurotrophin receptors (24). However, neurotrophin (e.g., NGF) receptors also are implicated in mediating hyperalgesia in delayed-onset muscle soreness (48). Further studies should probe whether the antinociceptive effects of SP are blocked by neurotrophin receptor antagonists.

Another surprise of the study was that activation of an M-channel-like current contributed to I_{SP-O} in muscle-afferent DRG neurons. Many GPCRs with G proteins G_q/G_{11} (including the NK1 receptor) are known to close M channels (Kv7) in neurons of both the peripheral and central nervous systems, although the underlying mechanisms still are not clear (49). The SP-mediated M-channel-like activation is intriguing, because inhibition of the M current in nociceptors may be one of the general mechanisms underlying pain produced by inflammatory mediators (50, 51).

Although an antinociceptive role for SP is not a new idea, this concept has been almost ignored for decades. In early studies, SP at low doses could release endorphins in the brain and thus mediate analgesia (52, 53). However, the SP analgesia occurred only when tested animals were hypersensitive to noxious stimuli (54). Therefore, the SP analgesia was interpreted as an action of normalizing responsiveness to pain. In contrast, we revealed SP antinociception for acid pain in peripheral sites of muscle nociceptors. Intriguingly, the effect of endogenous SP release failed during the second acid challenge. One possible explanation is that muscle nociceptors become more resistant to SP after i.m. acid challenge, perhaps because of the SP-mediated Src activation, which could interact with β -arrestins and affect GPCR trafficking and down-regulation, although the involvement of other proton-sensing GPCR signaling is possible also (55, 56). Further studies should probe how the first acid challenge alters SP–NK1 signaling in a delayed phase.

Increased SP levels often are associated with chronic muscle pain. Most patients with fibromyalgia show SP levels in cerebrospinal fluid above the highest normal control value (57). In patients with continuous idiopathic cervical pain, muscle tissues associated with myofascial trigger points show higher SP levels and lower pH values than do muscle tissues with latent myofascial trigger points in patients with no neck pain or normal controls (58). Likewise, increased SP immunopositive muscle-afferent neurons are found in rats with muscle inflammation (18, 59). However, the biological relevance of the increased SP levels has never been examined in detail. Here, we suggest that chronic muscle pain might elicit increased SP as a feedback control system counteracting the sensitization of muscle nociceptors and the acid-induced pain. Given the specific antinociceptive effect of SP on muscle nociceptors, NK1 receptor antagonists might worsen the muscle pain condition and thus compromise their clinical efficacy in treating pain (41, 60). Our finding offers insights for ongoing clinical trials testing NK1 antagonists in fibromyalgia patients (61). In particular, blocking SP–NK1 signaling might increase the risk of muscle-originated chronic hyperalgesia. In contrast, using SP as a peripheral analgesic might be conceivable, although SP (in the micromolar range) is equally potent as a spinal-sensitizing mediator in vitro (5, 62). Local application of SP could inhibit the excitability of muscle nociceptors effectively, and the local SP dosage would be largely diluted and have little effect on nociceptive transmission in the spinal cord.

Materials and Methods

Animals. We used adult (8- to 12-wk-old) C57/BL6 mice. All procedures followed the *Guide for the Use of Laboratory Animals* (National Academies Press, Washington, DC) and were approved by the Institutional Animal Care and Use Committee of Academia Sinica. We aimed to minimize the number of animals used and their suffering without compromising the quality of the experiments. $Tac1^{-/-}$ mice (40) and $Asic3^{-/-}$ mice (63) were generated and genotyped as described. Both null-mutant mice were backcrossed to C57/BL6 mice for 10 generations to establish a congenic strain. Congenic $Tac1^{-/-}$ $Asic3^{-/-}$ mice were offspring of $Tac1^{-/-}$ and $Asic3^{-/-}$ intercrosses.

Behavioral Assays. The mouse model of chronic mechanical hyperalgesia induced by repeated i.m. acid injection was modified from the Sluka et al. model (*SI Material and Methods*) (13).

Plasma Extravasation. Extravasation levels were evaluated by quantifying Evans blue staining in tissues as described (*SI Material and Methods*) (64).

DRG Primary Culture. DRG neurons of all lumbar segments were cultured on poly-L-lysine-coated cover slides as previously described (*SI Material and Methods*) (30, 65).

Whole-Cell Patch-Clamp Recording. Details of whole-cell patch-clamp recording are given in *SI Material and Methods*.

Data Analysis. Results are presented as mean \pm SEM and were analyzed by use of Origin 8.0 (OriginLab). One-way ANOVA and then a Fisher least significant difference post hoc test were used to calculate differences between groups (Fig. 2). Other electrophysiological data were analyzed by paired or unpaired Student's *t* test as appropriate. The Mann–Whitney *u* test was used to compare withdrawal responses to von Frey filament in mice before acid injection (baseline) and at each time point after i.m. acid injection. $P < 0.05$ was considered statistically significant.

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