Reactive oxygen species are second messengers of neurokinin signaling in peripheral sensory neurons

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Substance P (SP) is a prominent neumodulator, which is produced and released by peripheral damage-sensing (nociceptive) neurons; these neurons also express SP receptors. However, the mechanisms of peripheral SP signaling are poorly understood. We report a signaling pathway of SP in nociceptive neurons: Acting predominantly through NK1 receptors and G\(\alpha_q\)/\(\alpha_i\) proteins, SP stimulatres increased release of reactive oxygen species from the mitochondrial electron transport chain. Reactive oxygen species, functioning as second messengers, induce oxidative modification and augment M-type potassium channels, thereby suppressing excitability. This signaling cascade requires activation of phospholipase C but is largely uncoupled from the inositol 1,4,5-trisphosphate sensitive Ca\(^{2+}\) stores. In rats SP causes sensitization of TRPV1 and produces thermal hyperalgesia. However, the lack of coupling between SP signaling and inositol 1,4,5-trisphosphate sensitive Ca\(^{2+}\) stores, together with the augmenting effect on M channels, renders the SP pathway ineffective to excite nociceptors acutely and produce spontaneous pain. Our study describes a mechanism for neurokinin signaling in sensory neurons and provides evidence that spontaneous pain and hyperalgesia can have distinct underlying mechanisms within a single nociceptive neuron.

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sensitization of CAP responses, although with lower efficacy and affecting a smaller proportion of DRG neurons (22 and 17%, respectively) (Fig. 1C). These experiments suggest that (i) at least 40% of small, TRPV1+ DRG neurons express functional NK1, and (ii) NK2 and NK3 also are expressed in some TRPV1+ DRG neurons, but NK1 predominates; moreover, because the percentage of SP- and Sar-met SP-responsive neurons is the same, NK2- and NK3-expressing neurons also express NK1. A similar proportion (23/51, 45%) of TRPV1+ TG neurons displayed sensitization of CAP responses by SP (Fig. 1D). RT-PCR experiments (Fig. S1) supported the findings that both DRG and TG neurons express all three NKR and that NK1 is the predominant mRNA in both types of ganglia.

NKR were suggested to couple to a Goq11 signaling cascade (5, 6); this coupling involves hydrolysis of membrane PIP2 with subsequent activation of IP3–Ca2+ and DAG–PKC pathways. Sensitization of CAP responses by SP was suggested to be mediated by the action of PKC on TRPV1 (7, 8), suggesting that DAG is released upon SP application. Therefore, we next determined whether the IP3–Ca2+ branch of the Goq11 signaling cascade is activated also. First, we transfected DRG neurons with the PIP2/IP3 optical biosensor PLC6-PH-GFP to evaluate IP3 hydrolysis by PLC and the release of IP3. This probe is localized to the plasma membrane at rest and translocates to the cytosol following cleavage of IP3 from PIP2. In 9/34 (26%) small DRG neurons we observed significant translocation of the probe, indicating IP3 hydrolysis; i.e., the application of SP resulted in a 1.9 ± 0.3-fold increase in cytosolic fluorescence in these neurons (Fig. 2A and B). The magnitude of PLC6-PH-GFP translocation induced by SP was comparable to that induced by another Goq11 GPCR, the bradykinin (BK) B2 receptor, in similar conditions (12). Unexpectedly, however, SP- and NKR-specific agonists generally were unable to induce elevations of intracellular calcium ([Ca2+]i) (Fig. 2C). Only 43/336 (13%) of TRPV1+ DRG neurons responded to 1 μM SP with small Ca2+ transients (Fig. 1C), a population significantly smaller than that which displayed SP-induced potentiation of CAP responses (P < 0.001, χ2 test). Similarly, poor coupling between NKR and ER Ca2+ stores was observed in TG neurons (Fig. 1D). SP-induced Ca2+ transients in DRG and TG were markedly smaller than those induced by BK (P < 0.001) (Fig. 2C–E). These experiments suggest that (i) NKR activate PLC (shown by translocation of the IP3-sensitive optical probe) and PKC (shown by sensitization of CAP responses); (ii) NKR are functionally disconnected from the ER Ca2+ stores, resulting in a lack of cytosolic Ca2+ rise in response to SP in the majority of NKR-expressing neurons; and (iii) the few neurons in which SP does induce Ca2+ transients respond with much weaker Ca2+ signals than would be expected from a Goq11 agonist.

Fig. 1. Functional expression of NKR in sensory neurons. (A and B) Ca2+ imaging in small-diameter TRPV1+ DRG neurons loaded with fura-2 AM. (A) Capsaicin (CAP, 50 nM) was added to the bathing solution as indicated by the black bars. Before the fourth application of CAP, an NKR agonist was added to the bathing solution as indicated by the orange shaded area. *CAP MAX* indicates a saturating dose of capsaicin (1 μM). Each trace represents an example of the effect of different NKR agonists; the key is shown in B. All NKR agonists were applied at a concentration of 1 μM. SP is an agonist of all three NKR; Sar-met SP is an agonist of NK1, β-ala NKA is an agonist of NK2; and senktide is an agonist of NK3. Data are presented as the fluorescence ratio (340/380 nm, R) normalized to the initial ratio at time = 0 s (R0). (B) Mean data from A normalized to the size of the first CAP peak response (CAP1). Only cells that showed sensitization of the CAP4 response were included. ***Significant difference between CAP3 and CAP4 peak response (P < 0.001; paired t test). (C) Proportion of DRG neurons responding with a rise in cytosolic Ca2+ in response to NKR agonists (Left) or with a sensitization of TRPV1 (Right). Significant difference in the proportions of Ca2+ responders and TRPV1 sensitizers is shown by ∗∗∗P < 0.01 and ∗∗∗∗P < 0.001 (χ2 test). Ca2+ responders: SP, n = 43/336; Sar-met SP, n = 20/435; β-ala NKA; NK2, n = 28/228; senktide, n = 7/218. TRPV1 sensitization: SP, n = 32/83; Sar-met SP, n = 46/121; β-ala NKA, n = 51/233; senktide, n = 36/218. (D) As in C but using TG neurons and fluo-4 as the Ca2+ indicator dye. Ca2+ responders: SP, n = 39/200; Sar-met SP, n = 10/124; β-ala NKA, n = 15/124; senktide, n = 18/124. TRPV1 sensitization: SP, n = 23/51; other NKR agonists were not tested in TG neurons.

Fig. 2. NKR triggering in sensory neurons induces PLC activation but does not induce strong Ca2+ release from intracellular stores. (A and B) Translocation of the PIP2/IP3 probe PLC6-PH-GFP in the transfected DRG neuron in response to SP. (A) Low-resolution epifluorescence image of the transfected DRG neuron (Upper Left). (Scale bar, 100 μm.) Other images are confocal micrographs of the same neuron before (basal), during (SP), and after (wash) application of 1 μM SP; the neuron shown is representative of 9/34 cells tested. (Scale bars, 10 μm.) (B) Time course of the cytosolic fluorescence intensity measurements from the cell shown in A. (C) Sample trace showing the relative size of Ca2+ transient elicited by SP (1 μM) or BK (1 μM) in DRG neurons measured using fura-2 AM. (D and E) Mean data from experiments in C for DRG neurons. Number of cells is stated inside bars. ∗∗∗Significant difference between groups (P < 0.001; unpaired t test). (E) As in D, but for TG neurons measured using Fluo-4.
majority (39/46) of DRG neurons that responded to SP with Ca\(^{2+}\) rises also responded to CAP. A similar trend was observed in TG neurons, although slightly more TG than DRG neurons responded to SP with Ca\(^{2+}\) transients (39/200, 19.5\%, vs. 43/336, 13\%; \(P \leq 0.05\), \(\chi^2\) test) (Fig. 1 C and D).

**SP Augments M Current in Sensory Neurons via Oxidative Modification.** The finding that some G\(_{\text{q/11}}\) receptors do not induce release of Ca\(^{2+}\) from the endoplasmic reticulum (ER) is not unprecedented; for example, in sympathetic neurons muscarinic acetylcholine (M\(_3\)) receptors robustly activate PLC and hydrolyze PIP\(_2\), but this action does not result in ER Ca\(^{2+}\) release, presumably because of poor spatial coupling between the receptors and the PIP\(_2\)-sensitive stores (15–17). Nevertheless, these M\(_3\) receptors exert a robust excitatory effect in sympathetic neurons by inhibiting M current via PIP\(_2\) depletion (18, 19). DRG neurons also express M channels [K\(\text{V}7.2\), K\(\text{V}7.3\), and K\(\text{V}7.5\) (20, 21)], and M-channel inhibition produces strong excitatory effects in DRG (12, 20, 22, 23). Therefore, we tested whether SP inhibits M current in DRG and TG neurons. We performed patch-clamp recordings from small DRG and TG neurons (whole-cell capacitance of 28 ± 2 pF, \(n = 32\) and 26 ± 1 pF, \(n = 63\), respectively) that were responsive to CAP. Surprisingly, in a large proportion of DRG and TG neurons, SP induced marked augmentation of M current (Fig. 3A). In 25/43 DRG and 14/33 TG neurons SP induced a significant increase in the M-current amplitude of 59 ± 14\% and 81 ± 19\%, respectively (\(P < 0.001\); ANOVA). In 1/43 DRG and 4/33 TG neurons SP inhibited M current to 88\% and 32 ± 7\% of basal levels, respectively, whereas other neurons showed no response (presumably because they lacked functional NKR) (Fig. 3 A–D). Consistent with previous data (20), most of the small, CAP-responsive DRG and TG neurons expressed M current (e.g., 17/20 DRG neurons in one cohort). The amplitudes of deactivating current (I\(_{\text{deac}}\)) at −60 mV in DRG and TG neurons were 85 ± 8 pA (\(n = 32\)) and 43 ± 5 pA (\(n = 63\)), respectively.

Current-clamp recordings showed that SP failed to produce an excitatory effect in all 17 DRG neurons tested; each fired a single action potential (AP) before and after the SP application (Fig. 3E). In neurons that responded to SP with M-current augmentation, SP induced a moderate hyperpolarization of the resting membrane potential (compared with the population of neurons in which SP did not affect M-current amplitude) (Fig. 3E and F). The threshold for AP firing was not altered significantly, although there was a trend toward an increase (\(P = 0.177\)) in cells that responded to SP with an increase in M current (Fig. 3G).

The augmentation of M current induced by SP was slow (>10 min) and was not reversible upon washout, features that are reminiscent of augmentation of recombinant Kv7 channels by oxidative modification caused by H\(_2\)O\(_2\) (24). H\(_2\)O\(_2\) oxidizes a triplet of cysteines in the cystolic S2–S3 linker of Kv7 channels, an effect reversed by the reducing agent DTT (24). We therefore tested if DTT (1 mM) would reverse the M-current augmentation by SP, and indeed it did so (Fig. 4A and B). H\(_2\)O\(_2\) also augmented M current in DRG neurons, and, again, this action was reversed by DTT (Fig. 4C and D). In both cases DTT did not inhibit M current completely but rather returned the M current amplitude to near the basal level. DTT alone did not affect M current amplitude; moreover, DTT pretreatment rendered SP ineffective in producing M-current augmentation (Fig. 4B). These data suggest that M-current augmentation produced by SP is mediated by a mechanism similar to that produced by external application of H\(_2\)O\(_2\). Oxidation of Kv7.2/7.3 channels overexpressed in CHO cells with H\(_2\)O\(_2\) was shown to produce an acceleration of channel activation and a modest slowing of deactivation kinetics (24, 25). In DRG neurons the kinetics of M current was difficult to analyze because of contamination with other voltage-gated conductances. We fit the activation kinetics with a double-exponential function; the fast component was contaminated with conductances other than M current, but the slow component of activation (\(\tau_{\text{slow}}\)) was comparable with the kinetics of recombinant Kv7.2/Kv7.3 channels (24, 25).

Fig. 3. SP augments M current and increases the AP firing threshold in small-diameter sensory neurons. (A–C) Sample perforated patch-clamp recordings from TG neurons. M current is plotted as the magnitude of the small-diameter sensory neurons. (A) Current-clamp recordings from a DRG neuron. The effect of SP is shown after 15 min exposure.

**Inset** shows current traces recorded at the time points indicated (1–4). (D) Proportion of DRG and TG neurons responding with an increase, decrease, or no effect in M current in response to SP. The number of cells is shown within the pie charts. (E) Whole-cell current-clamp recording from a DRG neuron. The effect of SP is shown after 15 min exposure. **Inset** shows the current injection protocol. (F and G) Changes in membrane voltage (\(V_m\)) (F) and AP firing threshold (G) after 15-min bath perfusion of SP (1 \(\mu\)M). Each point represents one experiment (\(n = 17\)). **Significant difference between groups (\(P < 0.01\); unpaired t test).
neurons in the field. To identify the source of the SP-induced ROS generation in DRG neurons, we used mitochondrially targeted fluorescent protein, mt-cpYFP, which is particularly sensitive to superoxide anion (O$_{2}^{−}$·) but is insensitive to Ca$^{2+}$, ATP/ADP, and NAD(P)H (28). Importantly, the fluorescent signal is reversible, and brief flashes of fluorescence can be visualized in mitochondria during release of O$_{2}^{−}$· (28). Fig. 5B, Upper depicts a DRG neuron successfully transfected with mt-cpYFP. The probe was distributed heterogeneously within the cell, suggesting compartmentalization consistent with a mitochondrial localization. One micromolar SP (1 μM) induced a small but significant (P ≤ 0.05) increase in total cellular fluorescence in 5/10 small neurons (Fig. 5C; initial fluorescence rundown reflects GFP photobleaching because of the high sampling rate necessary to resolve individual flashes; see below). In three of five neurons we were able to resolve bright, localized flashes (Fig. 5B) that showed kinetics similar to the mitochondrial O$_{2}^{−}$· flashes reported previously using the same probe (28). Taken together, these experiments strongly suggest that SP induces mitochondrial ROS release in a subpopulation of small DRG neurons and reinforce our hypothesis that SP-induced augmentation of M current in DRG and TG neurons is mediated by ROS.

Mitochondrial superoxide production is dependent on electron transport chain (ETC) activity (28); inhibition of the ETC complex III with antimycin A has been shown to cause a burst of mitochondrial ROS release (29), whereas the protonophore and mitochondrial uncoupler N,N,N,N’-tetramethyl-p-phenylenediamine dihydrochloride (FCCP) prevents mitochondrial ROS release (28). Therefore, we tested if these compounds would interfere with the ability of SP to augment M current in DRG neurons. Antimycin A (25 μM) caused a 25 ± 10% augmentation of the M-current amplitude in five of nine small DRG neurons (Fig. 6 A and B), whereas 1 μM FCCP prevented M-current augmentation by SP in nine of nine DRG and four of four TG neurons tested; in fact in the presence of FCCP, M current was inhibited in seven of nine DRG and four of four TG neurons by 47 ± 7% and 82 ± 7%, respectively (P < 0.001) (Fig. 6 C and D). This inhibition probably was caused by the unmasking of PLC-mediated inhibition of M current (e.g., by PIP$_{2}$ hydrolysis and, in some cells, by small Ca$^{2+}$ transients).

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To probe if SP has a direct effect on the mitochondrial ETC in DRG neurons, we measured the oxygen consumption and oxidative phosphorylation rates in suspensions of freshly dispersed DRG using respirometry. Application of SP suppressed the mitochondrial oxygen consumption rate significantly, by $13 \pm 2.1\%$ ($n = 5$) (Fig. 6E). This finding was consistent with the previous experiment in which the complex III inhibitor, antimycin A, mimicked the M current-enhancing effect of SP. We therefore repeated the respirometry experiment using an extracellular solution containing the complex I inhibitor, rotenone (1 $\mu$M), and 10 mM succinate in place of glucose. Such conditions exclude complex I from ETC flux but permit respiration, because electrons from succinate oxidation enter the ETC directly via complex II (succinate dehydrogenase). In such conditions, SP still reduced mitochondrial respiration significantly (Fig. 6F). Because ETC complexes I and III are the main sources of mitochondrial ROS generation (50), it is likely that SP signaling affects complex III.

**NKR Couple to G$_{i/o}$ in Sensory Neurons but Can Couple to G$_{q/11}$ When Overexpressed.** Clearly, the SP-induced signaling cascade in small sensory neurons differs significantly from the classical G$_{q/11}$-mediated signaling cascade; so the question arises: Which G protein $\alpha$-subunit mediates the effect? Pertussis toxin (PTX)-sensitive G$_{i/o}$-coupled receptors, such as somatostatin receptors, were reported to augment M current in hippocampal neurons (31, 32) via an unidentified mechanism [although the involvement of arachidonic acid metabolites has been suggested (33, 34)]. Thus, we tested if the effect of SP in sensory neurons also is mediated by G$_{i/o}$. After treatment with 300 ng/mL PTX overnight, SP failed to augment M current in five of five TG and 9/10 DRG neurons (Fig. S3A). The proportion of small neurons responding to SP with Ca$^{2+}$ transients also was reduced significantly after PTX treatment; only 4/148 (3%) DRG and 5/120 (4%) TG neurons responded to SP with small Ca$^{2+}$ elevations, and these elevations were significantly smaller than in control DRG and TG cultures ($P \leq 0.001$ with $\chi^2$ test) (Fig S3 B and C). Interestingly, SP-mediated M-current augmentation in DRG neurons was blocked by the PLC inhibitor edelfosine (10 $\mu$M): Nine of nine edelfosine-treated neurons showed no augmentation (Fig S3 D). These experiments suggest that in DRG and TG neurons the effect of SP is mediated by G$_{i/o}$ protein and a PLC isoform that is not coupled to G$_{q/11}$ exclusively but also can be activated by G$_{i/o}$ [e.g., PLC$_{\beta 2}$ or $\beta_3$ (35)].

These results were unexpected, given that in other tissues and expression systems NKR were shown to couple to G$_{q/11}$ (5, 6). Thus, we expressed NK1–3 receptors in CHO cells and tested major components of the G$_{q/11}$ pathway by (i) confocal imaging of PIP$_2$ hydrolysis and IP$_3$ release using PLC6-PH-GFP; (ii) confocal imaging of DAG release with the PKCγ-C1-GFP probe; (iii) fura-2 Ca$^{2+}$ imaging to monitor Ca$^{2+}$ release; and (iv) patch-clamp recording of recombinant M-channel inhibition by NKR. NK1 (stimulated with 1 $\mu$M SP) induced robust translocation of PLC6-PH-GFP into the cytosol (Fig. 7A) and PKCγ-C1-GFP to the plasma membrane (Fig. 7B). Likewise, all NKR induced robust Ca$^{2+}$ transients (Fig. 7C), which were not affected by PTX pretreatment. We also measured the effect of recombinant NK1 on recombinant M channels (Kv7.2/7.3 heteromers) and found that 1 $\mu$M SP acutely inhibited M current by 50 ± 7% ($P \leq 0.001$; $n = 10$) (Fig. 7D). Taken together, these data indicate that, when overexpressed in CHO cells, NKR can couple to a classical G$_{q/11}$ signaling cascade. Although SP signaling in sensory neurons is different, it does induce PIP$_2$ hydrolysis (as evidenced by the PLC6-PH-GFP translocation and edelfosine blockade of M-current augmentation). This hydrolysis is likely to be spatially discrete from M channels (which are inhibited by PIP$_2$ depletion) and from IP$_3$-sensitive ER stores, because NKR rarely induced M channel inhibition or ER Ca$^{2+}$ release. To test if this endogenous NKR coupling rule can be overcome, we overexpressed NK1 in DRG neurons (Fig. 7 E and F) and tested if such overexpression would affect NK1 signaling. In DRG neurons overexpressing NK1, as in NK1-transfected CHO cells, SP induced robust Ca$^{2+}$ transients in eight of eight neurons (R/R$_0 = 2.07 \pm 0.34$, $n = 8$) (Fig. 7E) and also robustly and reversibly inhibited M current (Fig. 7F). Thus, we conclude that, when overexpressed in CHO cells or DRG neurons, NK1 receptors couple to G$_{q/11}$, but endogenous NK1 receptors in sensory neurons preferentially signal through G$_{i/o}$.
the effect of SP on the behavioral manifestations of nociception by using pain tests and on CGRP release from intact DRG neurons in culture by ELISA. As expected, both CAP (100 nM) and BK (1 µM) induced robust CGRP release, consistent with the excitatory effects of both agents. In contrast, 1 µM SP not only failed to stimulate CGRP release but significantly inhibited basal release (Fig. S4), consistent with the moderate hyperpolarization induced by SP in current-clamp experiments.

In a recent study (12) we analyzed excitatory and proalgesic effects of BK in sensory neurons and concluded that the acute “spontaneous” excitation (and the acute phase of BK-induced pain) is mediated mostly by the cytosolic Ca²⁺-transients, which inhibit M current and activate Ca²⁺-activated Cl⁻ channels (CaCC) encoded by Ano1/Tmem16a [in peripheral sensory neurons the [Cl⁻], is high, so activation of a Cl⁻ channel produces depolarization (12, 36)]. At the same time, it is postulated that thermal hyperalgesia induced by BK is mediated by the sensitization of sensory TRP channels [e.g., TRPV1 (37) and TRPA1 (38)]. Present data suggest that peripheral NKR are largely uncoupled from Ca²⁺ transients. Consistently, we did not observe activation of CaCC by SP (Fig. S5). Nevertheless, NKR do enhance Ca²⁺ responses to CAP in DRG, suggesting TRPV1 sensitization (Fig. 1). We therefore tested the peripheral effects of SP on the excitability of nociceptive neurons in vivo and compared them with the effects of BK. First we confirmed the previously characterized (7, 39) hyperalgesic effect induced by plantar injection of SP. In accordance with previous studies, the latency of hind paw withdrawal at the presentation of a thermal stimulus was decreased significantly after plantar injection of 10 µM SP in saline (50 µL; 0.5 nmol per site) (Fig. 8D); the hyperalgesic effect of SP was comparable to that of 10 µM BK (Fig. 8A). Equal concentrations of SP and BK were used, because the potency of BK at B₂ receptors and SP at NK₁ receptors are comparable in neurons and both have an EC₅₀ in the low nanomolar range (12, 40). Remarkably, in the nocifensive behavior test (total time of paw biting, licking, and flinching) BK, but not SP, produced prominent spontaneous pain (Fig. 8B). The lack of SP-induced spontaneous pain is consistent with previous observations in humans (41) and with the current study showing poor coupling of NKR to the IP₃–Ca²⁺ route of the PLC pathway along with a lack of M-current inhibition (and CaCC activation) in DRG neurons.

**Effects of SP on CGRP Release and Nociception.** Excitation of peripheral afferents induces nociceptive signaling to the CNS and also a local release of neuropeptides. Therefore, we evaluated the effect of SP on the behavioral manifestations of nociception by using pain tests and on CGRP release from intact DRG neurons in culture by ELISA. As expected, both CAP (100 nM) and BK (1 µM) induced robust CGRP release, consistent with the excitatory effects of both agents. In contrast, 1 µM SP not only failed to stimulate CGRP release but significantly inhibited basal release (Fig. S4), consistent with the moderate hyperpolarization induced by SP in current-clamp experiments.

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Discussion

Here we describe an atypical signaling cascade mediated by SP in sensory neurons. We show that (i) nociceptive DRG neurons express functional NKR1–3, but NK1 predominates, and neurons expressing NK2 and NK3 most likely also express NK1; all three receptor types are capable of potentiating CAP responses. (ii) When overexpressed in CHO cells or sensory neurons, NKR couple to the G_q11 cascade, but native NKR in DRG neurons signal predominantly through a PTX-sensitive protein (presumably Go_q). This signaling involves activation of PLC (potentially PLC_C, or β3, which can be activated by G_q(q) binding) and hydrolysis of PIP2, but the magnitude or location of PIP2 depletion and IP3 release is not sufficient to produce inhibition of Kv7 channels or release of Ca^{2+} from the ER. (iii) In sensory neurons NKR activation results in mitochondrial ROS production and release because of the modulation of mitochondrial ETC (most likely at complex III). (iv) ROS act as second messengers to augment M current via oxidative modification of Kv7 channels: this M-channel augmentation has a moderate anti- excitatory outcome (as evident from hyperpolarization and inhibition of basal CGRP release by SP). (v) SP signaling in DRG and TG neurons generally is similar. However, there is significantly better coupling between NKR and IP3-sensitive Ca^{2+} stores in TG neurons. In sum, the signaling cascade used by endogenous NKR in sensory neurons is substantially different from the classical G_q11 signaling cascade, although it does share some features of the classical cascade (e.g., involvement of PLC, TRPV1 sensitization); exogenously expressed NKR do couple to the typical G_q11-PLC cascade.

Although it was assumed that NKR in DRG neurons couple to the G_q11 cascade (5, 6), cross-talk with other G proteins [e.g., G_{i123} (42), G_{lo} and G_{i3} (43)] in expression systems has been suggested elsewhere. We also suggest that NKR can couple to multiple classes of G proteins thereby providing a level of signaling diversity dependent on local G protein association. A recent study demonstrated a functional coassembly between G_{i3}-coupled (serotonin 5HT_2A receptor 2AR) and G_{i3}-coupled (metabotropic glutamate receptor mGluR2) receptors in cortical neurons, which sets a dynamic G_q-G_i balance of the resulting heteromeric receptor (44). It is conceivable that NKR in DRG may coassemble with a G_i receptor, producing hybrid G_q-G_i signaling. Such potential coassembly may provide an explanation for G_{i11} coupling of overexpressed NKR1 in DRG, because there would not be enough endogenous G_i partners for coassembly.

There is a substantial difference in NKR signaling toward Kv7 channels in DRG neurons and the well-established action of muscarinic and BK receptors, which inhibit M channels either by PIP_2 depletion or by Ca^{2+}/calmodulin (or by a combination of both), producing acute excitation. Here we show that although NKR in DRG neurons do indeed activate PLC, they are incapable of producing either of the signals inhibiting M channels, instead resulting in mitochondrial ROS release that produces oxidative augmentation of Kv7 channel activity. The reasons for this remarkable difference in signaling subroutines of PLC-coupled GPCRs are yet to be established but most likely include restricted spatial clustering (microdomains) of receptors and their effector molecules and targets, as has been suggested elsewhere (17). Interestingly SP inhibited M channels in frog sympathetic neurons (45, 46), indicating further species and/or neuronal type-dependent heterogeneity in NKR signaling.

NK1 receptor activation has been associated previously with increased ROS formation, particularly in the respiratory (27) and immune systems (26). However, neither the underlying mechanism nor the source of increased ROS has been elucidated fully. We show that NKR activation in sensory neurons inhibits mitochondrial ETC, an action that increases ROS formation and release, presumably from complex III. We speculate that SP exerts an effect analogous to that of antmicycin A, but the exact mechanism linking NKR with ETC modulation in sensory neurons remains to be elucidated.

Previous studies of peripheral NKR signaling resulted in certain contention, but we believe that our study can resolve at least some of the controversies. (i) One study (47) found no immunohistochemical evidence for the expression of NK1 in peripheral sensory neurons; however, we show that functional effects of SP on peripheral neurons can be demonstrated both in vitro and in vivo. (ii) Although one study (9) reported SP-induced Ca^{2+} rises in cultured DRG neurons, other reports did not (7, 8). Our data suggest that Ca^{2+} rises induced by SP are small and are not displayed by the majority of SP-responsive neurons. (iii) The SP-induced, PKCε-mediated sensitization of TRPV1 was attributed to both NK1 (7) and NK2 (8). We show that DRG and TG express all three NKR isoforms, which produce similar effects, although NK1 predominates. (iv) Both hyperpolarization (48) and excitatory effects (49–51) of SP in DRG have been reported. In our hands SP produced a moderate hyperpolarization because of an increase in M current, whereas the depolarizing effect of SP (49, 50) could have been caused by differences in recording conditions (i.e., a high concentration of cAMP or cesium fluoride in pipette solutions used in these studies). A study performed on the actively dissociated DRG neurons reported an inhibition of the voltage-gated K_+ currents by SP in most small neurons. Therefore activation was observed in some others (51). However, during the protoelectric dissociation of DRG neurons, M current is inhibited by the protease-activated receptor PAR-2 (22) and also by high cytosolic Ca^{2+} levels (52) that are unavoidable in acutely dissociated neurons; therefore, the effect of SP on M current is most likely underestimated in acutely dissociated neurons. Recently SP has been reported to enhance an XE991-sensitive current in a proportion of muscle afferent neurons through an Src kinase-dependent, GPCR-independent mechanism (53). Although M-current enhancement by SP is consistent with the present study, the signaling cascade is not. Thus, we have shown clearly that SP-mediated enhancement of M current in DRG is PLC dependent and is inhibited by PTX, both supporting a role for GPCRs. Src also is widely reported to have an inhibitory effect on M channels; an effect mediated by phosphorylation of two tyrosines [e.g., Y67 and Y349 in Kv7.3 (54–56)].

We show that the evidence that supports the above possibilities suggest some further hypotheses. (i) There is a substantial difference in the message conveyed to peripheral nerve endings by BK (which is released by the inflamed or damaged tissue and signals inflammation) and SP (which is released by the sensory nerves themselves). It is conceivable that in nociceptive neurons NKR, in contrast to BK B_2 receptors, lack an acute excitatory component of the signaling cascade, in this case resulting in a self-perpetuating positive feedback loop. In contrast, the hyperpolarizing augmentation of M current induced by SP may provide a negative feedback loop limiting further release of SP, as suggested earlier (11). However, NKR still induce thermal hyperalgesia by sensitizing TRPV1; this hyperalgesia may help maintain the sufferer’s awareness of the ongoing inflammation. (ii) A significantly larger proportion of TG neurons displays coupling between NKR and IP_3-sensitive Ca^{2+} stores compared with DRG neurons. Accordingly, SP inhibited M current in a larger proportion of TG neurons. This inhibition [possibly in combination with other Ca^{2+}-dependent effects, e.g., activation of TMEM16a/CaCC (12)] would be expected to excite trigeminal fibers and induce nociceptive signaling. Thus, we hypothesize that the NKR action would be more painful in the orofacial area than in the rest of the body. (iii) Our data clearly demonstrate that spontaneous pain and hyperalgesia can be mediated by distinct molecular mechanisms. These findings are of particular importance for designing future strategies for analgesic drug discovery, because spontaneous pain and hyperalgesia are conceptually poorly distinguished at present.
Furthermore, although chronic pain, which causes most suffering to humans, is mostly spontaneous in nature, the major animal models for testing analgesic efficacy of drugs are based on hyperalgesia tests (57). Accordingly, despite the rapid progress in our understanding of the mechanisms of hyperalgesia, there has been limited achievement in treatment of pain in humans. Further studies highlighting the mechanistic difference in spontaneous pain and hyperalgesia are needed to provide a new framework for analgesic drug design.

Materials and Methods

Cell Cultures, Transfections, cDNA Constructs and Chemicals. DRG and TG neurons were extracted from 7-d-old rats, enzymatically dissociated, and cultured for at least 24 but not more than 96 h before experiments (as described in ref. 12); no NGF was added to the culture medium. CHO cells were cultured in DMEM/F12 medium. Plasmids encoding human Kv7.2 and human Kv7.3 (GenBank accession numbers, AF110020 and AAC96101, respectively) were given to us by David McKinnon (State University of New York at Stony Brook, Stony Brook, NY) and Thomas Jentsch (Zentrum fur Molekulare Neurobiologie, Hamburg, Germany) and were subcloned into pcDNA3.1 (Invitrogen). The PLC-δ-FP-GFP and PKCγ-C1-CFP constructs were kind gifts from Tobias Meyer (Stanford University, Palo Alto, CA). Human B2R, NK1, NK2, and Gq (GenBank accession number AY275455, AY462009, AY322545, and AY462099, respectively) were purchased from the Missouri Science and Technology cDNA Resource Center. The mitochondrial O2−• sensor, mt-cyFP, was a kind gift from Heping Cheng (Peking University, Beijing, China). CHO cells were transfected using FuGENE HD transfection reagent (Roche). DRG neurons were transfected using Amaxa Nucleofector Device (Lonza) in combination with the rat DRG transfection kit and O-03 i Positive Control Solution. Materials and Methods

Electrophysiology. Perforated patch-clamp recordings were performed as described previously (12, 22). The standard bath solution contained (in mM) 160 NaCl, 2.5 KCl, 1 MgCl2, 10 Hepes, and 8 glucose, pH 7.4. The standard pipette solution contained (in mM) 100 K-acetate, 10 KCl, 10 NaCl, 1 CaCl2, 3 MgCl2, 5 EGTA, 40 Hepes, and amphotericin B (250 µg/mL) pH 7.4. M-like current amplitude was measured from the deactivation current elicited by 600–800-ms square voltage pulses to −60 mV from a holding potential of −30 mV, calculated as the difference between the average of a 100 ms segment taken 20–30 ms into the hyperpolarizing step and the average during the last 50 ms of that step and termed “I∞−Vm” (22). The fraction of I∞−Vm attributable to M current was determined from the XE991-sensitive fraction and was termed “I∞M”. In current-clamp recordings, APs were generated by injection of 400-pA current for 1 s from a holding current of 0 pA. The AP firing threshold was determined from a train of current steps delivered in 10-pA increments. Fluorescence Imaging. Ca2+ imaging was performed as described previously (52). For ROS imaging with the CM-H2DCFDA dye, DRG neurons were loaded with the dye (5 µM) for 30 min and imaged with excitation with 488-nm light for 10 ms every 10 s. Excitation of the dye resulted in some time-dependent autofluorescence, which was monitored for the first 300 s and subtracted offline using a linear function. To measure mitochondrial O2−• release, DRG neurons were transiently transfected with mt-cyFP and imaged 48 h later using LiveScan Swept Field Confocal System (Nikon) with an 488-nm argon laser at three to five frames/s. Translocation of IP3/PIP2 and DAG probes was recorded using the confocal system as described previously (22); images were analyzed with NIS Elements 3.2 software (Nikon).

Respiratory Test. freshly dissociated rat DRG neurons were resuspended in the standard bath solution (see above) and transferred to the chambers of a high-resolution respirometer (Oxygraph-2K; Oroboros instruments). The chambers were sealed, and the oxygen consumption rate was measured at 37 °C by polarographic oxygen sensors. The sensors were calibrated each day immediately before the experiment. Oxygen flux (picomoles per second per 106 cells) was monitored continuously and allowed to reach a stable value (i.e., a constant rate of O2 consumption) before the addition of either 1 µM SP (giving a final concentration of 1 µM) or 1 µL vehicle to the chambers. Then the O2 flux was monitored until it was stable (typically 10–15 min). Oligo-mycin (2 µg/mL) was then added to inhibit complex V, and, when the flux was stable, the uncoupler FCCP was added, in 0.5-µM steps, allowing measurement of the maximum electron flux through the electronic transport system. Finally, nonmitochondrial respiration was measured after inhibition of complex III by 2.5 µM antimycin A. In one series of experiments glucose in the chamber solution was replaced by 10 mM succinate, and 1 µM rotenone was added to inhibit complex I of the electronic transport system.

Behavioral Assays. Wistar rats (body weight, 150 g) were grouped randomly and allowed to acclimatize for at least 20 min in a transparent observation chamber before the experiment. The right hind paw of the animal received an intraplantar 50-µL injection of 10 µM SP, 10 µM BK, or vehicle. Spontaneous pain behavior (time spent licking, biting, lifting, and flinching) was recorded using a video camera for 30 min. The videos were analyzed by an observer unaware of treatment allocations. For measurement of thermal hyperalgesia, the change in the latency of withdrawal in response to noxious heat was recorded using the Hargreaves’ plantar method (Ugo Basile). Baseline thermal latency was measured before a 50-µL injection of 10 µM SP, 10 µM BK, or vehicle, and measurements repeated at 10, 20, and 30 min after the injection. Both ipsilateral (injected) and contralateral (control) hind paw withdrawal latencies were recorded; neither BK nor SP had an effect on the contralateral paw. The power settings were kept constant throughout the series.

Statistics. All data are given as mean ± SE. Differences between groups were assessed by Student’s t test or ANOVA with Dunnett’s (one-way ANOVA) or Bonferroni (two-way ANOVA) post tests. The differences were considered significant at P ≤ 0.05. The χ2 test was used to determine differences in the number of cells responding to agonists.

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