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Substance P activates Mas-related G protein–coupled receptors to induce itch

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Abstract

Background—Substance P (SP) is linked to itch and inflammation through activation of receptors on mast cells and sensory neurons. There is increasing evidence that SP functions through Mas-related G protein–coupled receptors (Mrgprs) in addition to its conventional receptor, neurokinin-1.

Objective—Because Mrgprs mediate some aspects of inflammation that had been considered mediated by neurokinin-1 receptor (NK-1R), we sought to determine whether itch induced by SP can also be mediated by Mrgprs.

Methods—Genetic and pharmacologic approaches were used to evaluate the contribution of Mrgprs to SP-induced scratching behavior and activation of cultured dorsal root ganglion neurons from mice.

Results—SP-induced scratching behavior and activation of cultured dorsal root ganglion neurons was dependent on Mrgprs rather than NK-1R.

Conclusion—We deduce that SP activates MrgprA1 on sensory neurons rather than NK-1R to induce itch.

Keywords

Substance P; Mas-related G protein–coupled receptors; dorsal root ganglion neurons; calcium imaging; receptor antagonist; knockout mice

Recognition that itch is of major medical significance and poorly served by existing therapeutic options is driving research into the basic mechanisms that underlie this sensory phenomenon. Progress is being made in the identification of peripheral and central mediators, receptors, and channels that contribute to the sensation of itch and scratching

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behavior. Substance P (SP) has long been established as an inflammatory neuropeptide and potent endogenous pruritogen in mice and human subjects. The classic receptor for SP is the neurokinin-1 receptor (NK-1R). Several antagonists of this receptor have been developed to inhibit the nociceptive and proinflammatory properties of SP. These compounds are effective in animal models of several diseases but have proved to be surprisingly ineffective with respect to inflammatory diseases in human subjects. With respect to itch, there are both positive[1–3] and negative[4,5] reports on the effectiveness of NK-1R antagonists. It has been proposed that nerves are the main drivers of SP-induced itch in mice.[6] Recent studies have demonstrated that SP activates Mas-related G protein–coupled receptors (Mrgprs) in addition to NK-1R, but the interaction of SP with Mrgprs on nerves has not been evaluated. Here we demonstrate that SP activates Mrgprs on dorsal root ganglion (DRG) neurons and not NK-1R to induce itch.

Mrgprs on human and rodent small primary sensory neurons are implicated in nociception.[7] The concept of Mrgprs serving as innate environmental sensors in general and specifically as sensors for pruritogens is supported by a number of lines of evidence. Mrgprs first appear in tetrapods,[8] animals endowed with the capacity to remove exogenous agents by scratching while navigating on land. Mrgprs demonstrate high constitutive activity and respond to several ligands, many of which are pruritogens.[8–11] In addition, Mrgprs are expressed on peripheral terminals of sensory neurons and mast cells, which is consistent with a potential role in sensing.[8,9,12,13]

Recently, we identified an antagonist of human MRGPRX2 and the homologous mouse receptors MrgprA1 and MrgprB2.[14] This antagonist is termed Boc-Gln-D-Trp(Formyl)-Phe benzyl ester (QWF).[6] We also found that although SP-induced itch remained intact in NK-1R knockout (*NK-1R*$^{-/-}$) mice, QWF significantly decreased SP-induced itch in both wild-type and *NK-1R*$^{-/-}$ mice. Because QWF was found to inhibit SP-induced degranulation from mast cells, we suggested that SP-induced mast cell degranulation and SP-induced itch, at least in the periphery, is mediated through Mrgprs.[14] Although an interaction of SP with mast cells in the context of inflammation has long been established, SP-induced itch is not significantly decreased in mast cell–deficient mice.[6] A reasonable interpretation of these data is that SP interacts with a receptor on sensory nerves to evoke itch.[6]

Mrgprs are primarily expressed on DRG neurons and are implicated in itch and nociception.[15] Here we evaluated the interaction of SP with cultured murine DRG neurons. We deduce that MrgprA1 is responsible for SP-induced scratching behavior.

METHODS

Peptides and chemicals

SP was obtained from GenScript (Piscataway, NJ) and dissolved in 1× PBS. The NK-1R antagonist L733060 and the dual NK-1R/Mrgprs antagonist QWF (Boc-Gln-D-Trp[formyl]-Phe benzyl ester trifluoroacetate) were obtained from Sigma (St Louis, Mo). QWF and L733060 were dissolved in dimethyl sulfoxide (DMSO; Sigma) at 10 mmol/L and diluted in PBS for use at concentrations of 500 μmol/L or less. In the nociceptive studies with

capsaicin, in which QWF was diluted in DMSO, the final concentration of DMSO was 0.01%. Capsaicin was obtained from Tocris Bioscience (Bristol, United Kingdom).

Animals

Male and female *Mrgpr cluster* $\Delta^{-/-}$, *NK-1R*$^{-/-}$, TRPV1-Cre$^{+/-}$DTA$^{+/-}$ (which TRPV1^{+} sensory neurons are ablated[16]), or wild-type mice were used. Mice were 2 to 8 months old and weighed 20 to 30 g, and 4 to 7 were included per group, depending on the experiment.

Mrgpr cluster $\Delta^{-/-}$ mice on a C57BL/6 background were generously provided by Xinzhong Dong (Johns Hopkins, Baltimore, Md). *NK-1R*$^{-/-}$ mice on a C57BL/6 background were generously provided by Norma Gerard (Children's Hospital, Boston, Mass). The wild-type mice used for the scratching behavior experiments are littermates of the *Mrgpr cluster* $\Delta^{-/-}$ mice. The wild-type mice used for DRG culture and pain assessment and the TRPV1-Cre$^{+/-}$DTA$^{+/-}$[16] mice and their littermate controls were obtained from JAX (Jackson Laboratory, Bar Harbor, Me). All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital and Boston Children's Hospital.

Scratching behavior

The mouse cheek model was used in this study.[17] The animals were habituated for 30 min/d for 3 days before and 15 minutes on the day of the experiment. Using a BD insulin syringe (BD Biosciences, San Jose, Calif) with a 31-gauge needle, mice were injected intradermally with 10 μL containing SP (500 μmol/L) alone or in combination with L733060 (500 μmol/L) or QWF (500 μmol/L) into the right cheek. Mice were not shaved before injections to minimize irritation. All experiments were performed at consistent times during the day (9 AM to 2 PM). Mice were videotaped in a soundproof environment to minimize distraction. A blinded investigator scored recordings for the number of scratching bouts that occur over 1-minute intervals during 25-minute observation periods. A scratching bout was initiated by lifting the hind paw to the area of injection and ended by returning the hind paw to the floor or mouth. SP concentrations were selected based on the literature for behavioral tests.[6,14]

Pain assessment

The thermal latency threshold was assessed in wild-type mice by using the Hargreaves test (Plantar Test Apparatus; Ugo Basile, Comerio, Italy) 10 minutes after an intraplantar injection of 10 μL containing capsaicin (100 μmol/L prepared in DMSO) into the left hind paw.[18] A separate group of mice received a coinjection of 10 μL containing capsaicin (100 μmol/L) and QWF (1 μmol/L). The control group received 10 μL of the respective vehicle (DMSO).

DRG culture and calcium imaging

Cervical to lumbar DRGs from wild-type, *NK-1R*$^{-/-}$, TRPV1-Cre$^{+/-}$ DTA$^{+/-}$, and littermate control mice were dissected and pooled from groups of 4 mice and maintained in Dulbecco modified Eagle medium (Gibco, Grand Island, NY) containing 200 mmol/L L-glutamine (Fisher Scientific, Waltham, Mass), 10% heat-inactivated FBS (Gibco), 5000 U/mL penicillin, and 5000 μg/mL streptomycin (Fisher Scientific). Enzymatic digestion was

carried out with 1 mg of collagenase in 1 mL of Dispase (Roche Applied Sciences, Penzburg, Germany) at 37°C for 70 minutes. The collagenase/dispase solution was removed, and DRGs were washed and suspended in Dulbecco modified Eagle medium containing 125 U of DNAse (Sigma) within which the ganglia were mechanically triturated by using fire-polished glass pipettes. These cells were centrifuged over a 10% BSA solution (Sigma) gradient, pelleted at 1000 rpm, and suspended in Neurobasal medium (Gibco) supplemented with B27 (Invitrogen, Carlsbad, Calif), nerve growth factor (Invitrogen), glial cell–derived neurotrophic factor (Sigma), and arabinocytidine (Sigma). The cells were plated onto glass-bottom 35-mm dishes coated with 10 μg/mL laminin (Sigma) and cultured for 24 hours.

For calcium imaging experiments, neurons were loaded for 30 minutes with 10 μmol/L Fura-2 AM (Life Technologies, Grand Island, NY) in neurobasal medium, washed with standard extracellular solution (145 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 10 mmol/L HEPES, pH 7.5), and imaged at room temperature. Cells were evaluated with a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) equipped with an Exi Aqua CCD camera (QImaging, Surrey, British Columbia, Canada). Ca^{2+} flux fluorescence was measured as an absorbance ratio at 340 and 380 nm (F340/380; Lambda DG4; Sutter Instrument, Novata, Calif). The 340/380 ratiometric images were analyzed by using Nikon Elements AR Software (Nikon). SP (10, 30, 100, 300, and 1000 nmol/L) and QWF (1 μmol/L) solutions were delivered directly onto neurons at a flow rate at 2 mL/min for 20 seconds by using perfusion barrels, followed by buffer washout and further application. Allyl isothiocyanate (1 μmol/L; Sigma), 1 μmol/L capsaicin (Tocris Bioscience), and 40 mmol/L KCl (Sigma) were applied at the end of each experiment.

PCR of human DRG total RNA

Human DRG total RNA (from 16- to 65-year-olds) was purchased from Clontech Laboratories (Mountain View, Calif; catalog no. 636150) and converted into single-stranded cDNA by using random hexamers and reverse transcriptase with the SuperScript III First strand Synthesis kit (Invitrogen). PCR was carried out with cDNA as a template and primer pairs of hMRGPRX2: forward primers F1 (CTGTGATGACCTGTGCCTACCTTGC) and F2 (TCAGCGGTCGTGTGTGTCCTGCTCTGGG) and reverse primers R1 (CCGCCACTGCTTCCTAAAAGAGCCC) and R2 (GAAGAAGTAAATGATGGGGTTGGCAC). The primer pairs F1+R1 and F2+R2 yielded the expected PCR products 551 and 408 bp, respectively. PCR was carried out with New England Biolabs Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, Mass) at the following conditions: denaturing at 98°C, annealing at 55°C, and extension at 72°C.

Statistical analysis

Group data are presented as means ± SEMs of 6 to 7 animals depending on the study. This number of mice has been demonstrated by means of power calculation to generate significance in behavioral studies.[14] For statistical comparison, the 2-tailed paired or unpaired Student *t* test or 1-way ANOVA followed by the Tukey test was used to determine

significance. Differences were considered statistically significant at a *P* value of less than .05. Data analysis was performed with Prism 6 software (GraphPad, La Jolla, Calif).

RESULTS

SP-induced itch behavior is dependent on Mrgprs

We first investigated whether Mrgprs played a role in SP-induced scratching behavior. We used the mouse cheek model[17] to evaluate the pruritogenic effect of SP in *Mrgpr cluster* $\Delta^{-/-}$ mice in which 12 Mrgprs had been knocked out.[10] SP-induced itch was significantly decreased in these animals compared with that seen in wild-type mice (Fig 1, *A*). This result is consistent with previous studies suggesting a role for DRG neurons in addition to mast cells in patients with SP-induced itch.[6,14] To confirm these previous studies, we evaluated itch behavior in wild-type mice injected with SP together with L733060. L733060 is a widely used NK-1R antagonist that additionally blocks SP-induced activation of MrgprB2.[14] L733060 had no significant effect on SP-induced itch, implying minimal if any role for MrgprB2 in SP-induced itch (Fig 1, *B*). Furthermore, MrgprB2 is the only mouse Mrgpr expressed on mast cells and is intact in *Mrgpr cluster* $\Delta^{-/-}$ mice.[10,13]

In contrast, QWF, which is an antagonist of NK-1R, MrgprB2, and MrgprA1, but not other mouse Mrgprs,[14] inhibited SP-induced itch (Fig 1,*B*). MrgprA1 is expressed on sensory nerves but not mast cells.[19] This result is consistent with SP-induced itch being dependent on MrgprA1 on sensory neurons.

We next sought to demonstrate that the effect of QWF is restricted to itch responses. QWF exposure to cultured DRG neurons had no effect on the relative number of capsaicin and KCl-sensitive neurons or the amplitude of response induced by these stimuli (Fig 2, *A*, and see Fig E1 in this article's Online Repository at www.jacionline.org). QWF also did not affect capsaicin-induced thermal hyperalgesia *in vivo* (Fig 2, *B*). These findings are consistent with the activity of QWF on SP-induced itch being specific to the action of the ligand on these neurons.

SP activates DRG neurons from *NK-1R*$^{-/-}$ mice

The possibility that SP interacts with a receptor other than NK-1R on DRG neurons has not been reported. To explore this possibility, we evaluated the effect of SP on cultured DRG neurons from wild-type and *NK-1R*$^{-/-}$ mice. SP activated cultured DRG neurons from *NK-1R*$^{-/-}$ mice in a concentration-dependent manner (Fig 3, *A–C*). These activated cells were of small diameter and sensitive to capsaicin, which is consistent with TRPV1^{+} sensory neurons (Fig 3, *D*). This response was abolished in DRG cultures from TRPV1-Cre$^{+/-}$DTA$^{+/-}$ mice from which the TRPV1 sensory neurons have been ablated (Fig 3, *E*).[16] These results indicate that SP can activate pruriceptors through a receptor other than NK-1R.

QWF blocks SP-induced calcium flux in *NK-1R*$^{-/-}$ mice DRG neurons

To establish an *in vitro* correlate of the capacity of QWF to block SP-induced itch, we examined the capacity of QWF to prevent SP activation of DRG neurons from mice. QWF abolished SP-induced calcium flux in DRG neurons from *NK-1R*$^{-/-}$ mice (Fig 4). Repeated

treatment with SP was used to rule out the concern that the effects observed were due to desensitization. Such treatment led to similar calcium flux amplitude and numbers of responsive neurons (see Fig E2 in this article's Online Repository at www.jacionline.org). These data are consistent with QWF antagonism of SP-induced calcium flux in *NK-1R*$^{-/-}$ mice nociceptors, and we interpret this as showing that the peptide can act on these neurons independently of NK-1R.

DISCUSSION

The limited efficacy of antihistamines in most pruritic conditions[20,21] highlights the importance of histamine-independent mechanisms of itch. Mrgprs are among the most important mediators of histamine-independent itch.[22] This article further addresses the role of Mrgprs in itch by indicating a specific role for MrgprA1 in SP-induced itch in mice. SP has been implicated in various cutaneous and systemic pruritic disorders, such as atopic dermatitis[23,24] and cholestasis.[25] We have previously reported that wild-type and *NK-1R*$^{-/-}$ mice have comparable scratching behavior in response to SP and that *in vitro* SP activates MrgprA1 in addition to NK-1R. These findings are consistent with a role for a receptor other than NK-1R in the mediation of SP-induced itch responses.[14]

Here we show that SP-induced scratching behavior is dependent on Mrgprs because *Mrgpr cluster Δ*$^{-/-}$ mice demonstrate a significant reduction in the number of scratching bouts compared with wild-type animals. Our previous data on *NK-1R*$^{-/-}$ mice were confirmed by showing that when the NK-1R antagonist L733060 is injected with SP into wild-type mice, itch is not reduced, reconfirming in an independent fashion that NK-1R does not play a pivotal role in SP-induced itch in mice. In contrast, scratching behavior was reduced to baseline in animals cotreated with SP and the dual NK-1R/Mrgprs antagonist QWF, which is consistent with SP-induced itch being dependent on Mrgprs. These results are in line with data published by our group showing that QWF is an antagonist of MrgprA1.[14] Because MrgprA1 is the only Mrgpr in the cluster knockout mice to which QWF binds,[14] this specific receptor likely mediates SP-induced itch. To further test the hypothesis that SP induces itch through a receptor that is not NK-1R, we evaluated the ability of SP to activate DRG sensory neurons lacking NK-1R. As expected, SP excited *NK-1R*$^{-/-}$ DRG neurons that are of small size and TRPV1^{+} in a QWF-dependent fashion.

The data presented here favor a specific role for MrgprA1 on sensory neurons in SP-induced itch. It is appreciated that the availability of MrgprA1 sensory neuron conditional knockout mice would provide additional genetic-based clarity to our pharmacologic observations. Because QWF appears to have selectivity for itch over nociceptive responses (Fig 2 and see Fig E1), it is tempting to propose that we have uncovered a new receptor that specifically mediates SP-induced itch, and in consequence antagonists to a homologous receptor in human subjects might be useful for treating those pruritic disorders in which SP is involved, which we consider to be MRGPRX2.

MRGPRX2 is the only human Mrgpr expressed by mast cells.[13] Several endogenous and exogenous molecules activate MRGPRX2 to induce mast cell degranulation implicating the receptor in inflammation and pseudoallergic drug reactions.[13] The diversity of ligands

activating MRGPRX2 underscores its role as a sensor to detect a wide variety of stimuli. In addition to inflammation, a role for MRGPRX2 in itch has been proposed.[14,26] In line with this, MRGPRX2 is the only human Mrgpr activated by SP, an endogenous pruritogen.[14] Several studies have demonstrated expression of MRGPRX2 on human and primate DRG neurons by means of immunohistochemistry and PCR,[12,27,28] although one did not.[29] In an effort to address this discrepancy, we used PCR and amplified MRGPRX2 from RNA extracted from human DRGs (see Fig E3).

We have taken advantage of the different specificities of QWF and L733060 for antagonizing MrgprA1 and MrgprB2 to evaluate the roles of these receptors in SP-induced itch. Human MRGPRX2, which is expressed on mast cells and DRG neurons, functionally resembles MrgprB2 and MrgprA1 on mouse mast cell and DRG neurons, respectively. We conclude that MRGPRX2 is an important target for treating both mast cell–mediated pathologic actions and histamine-independent itch.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
Mrgpr	Mas-related G protein–coupled receptor
NK-1R	Neurokinin-1 receptor
QWF	Boc-Gln-D-Trp(Formyl)-Phe benzyl ester
SP	Substance P

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Clinical implications: Mrpgrs might be an important target for treating histamine-independent itch.

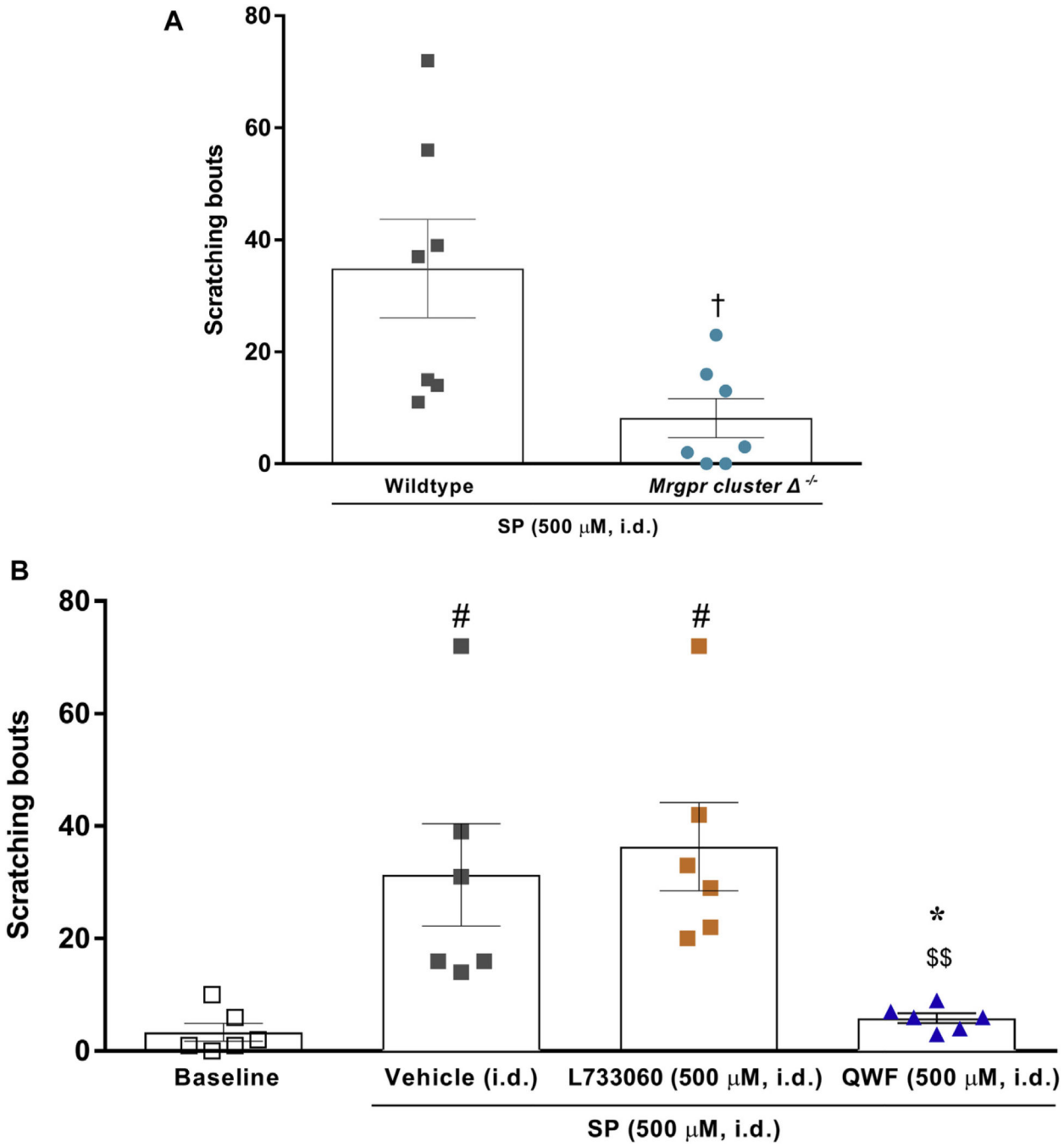

FIG 1.

Mrgprs mediate SP-induced itch. **A,** SP-induced itch (500 μmol/L administered intradermally *[i.d.]*) is significantly decreased in *Mrgpr cluster* $\Delta^{-/-}$ mice. **B,** Effects of treatment with the NK-1R antagonist L733060 (500 μmol/L administered intradermally) or the dual NK-1R/Mrgpr antagonist QWF (500 μmol/L administered intradermally) on scratching behavior caused by SP (500 μmol/L administered intradermally). Data are expressed as means ± SEMs. The unpaired Student *t* test (n = 7 mice per group in Fig 1, *A*) and 1-way ANOVA followed by the Tukey test (n = 6 mice per group in Fig 1, *B*) were used.

Significant differences from wild-type mice (†$P < .05$), baseline (#$P < .05$), SP plus vehicle (*$P < .05$), or SP plus L733060 ($$$P < .01$) are indicated.

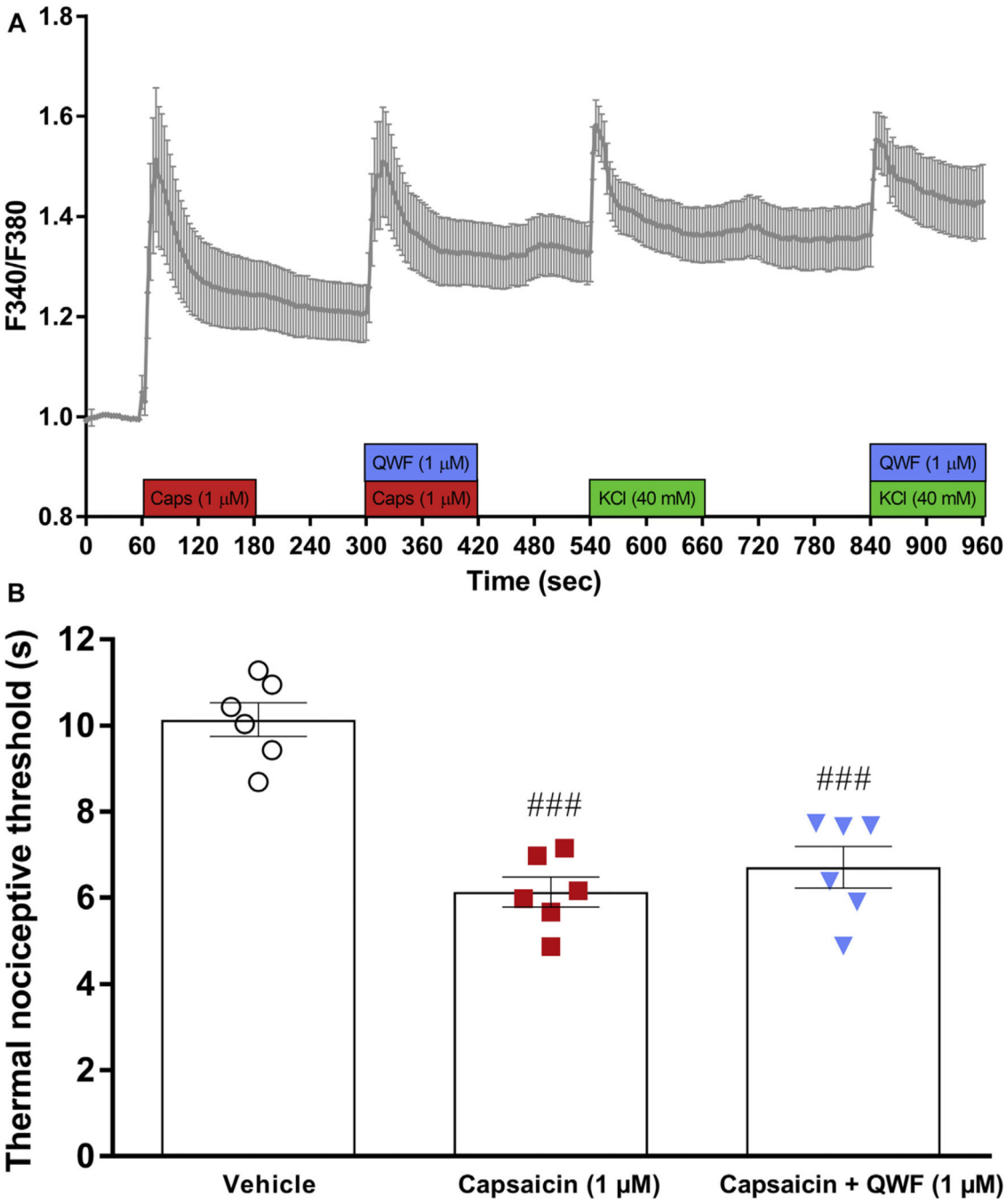

FIG 2.

Effect of QWF on capsaicin-induced Ca^{2+} flux and thermal hyperalgesia. **A,** QWF (1 μmol/L) does not affect Ca^{2+} flux induced by capsaicin (1 μmol/L) and KCl (40 mmol/L) in cultured wild-type DRG neurons. **B,** QWF (1 μmol/L, intraplantar) has no effect on capsaicin-induced thermal hyperalgesia in wild-type mice. Data are expressed as means ± SEMs. One-way ANOVA followed by the Tukey test (n = 6 mice per group). Statistical differences from vehicle are indicated as follows: ###$P < .001$. Total number of cells analyzed in Fig 2, *A*, was 267.

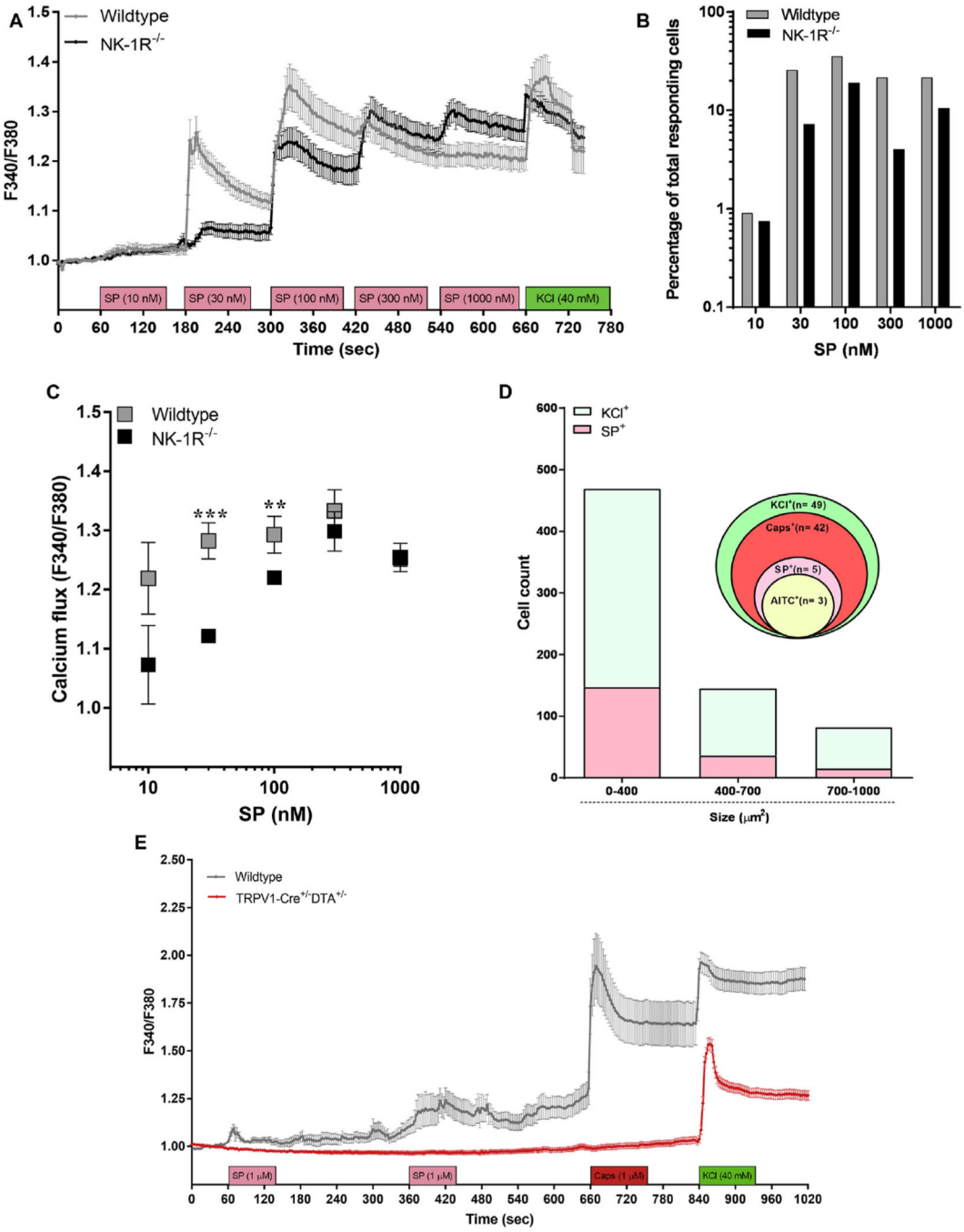

FIG 3.

SP activates *NK-1R*$^{-/-}$ nociceptors. **A,** SP (10–1000 nmol/L) induces Ca^{2+} flux in wild-type (gray) and NK-1R$^{-/-}$ (black) cultured DRG neurons. **B,** Percentage of SP-responding cells from wild-type or *NK-1R*$^{-/-}$ mice. **C,** Maximal Ca^{2+} flux in DRG neurons from wild-type (gray) and *NK-1R*$^{-/-}$ (black) mice. **D,** Size of SP-responsive cells from *NK-1R*$^{-/-}$ mice. *Inset*, Venn diagram showing overlapping populations of KCl-positive, capsaicin-positive, and SP-responsive neurons. **E,** Ca^{2+} flux induced by SP (1 μmol/L), capsaicin (1 μmol/L), and KCl (40 mmol/L) in DRG neurons from wild-type (gray) and TRPV1-

$Cre^{+/-}DTA^{+/-}$(red) mice. The unpaired Student *t* test was used. Significant differences from *NK-1R*$^{-/-}$ were indicated as follows: $^{**}P < .01$ and $^{***}P < .001$. Total number of cells analyzed was as follows: Fig 3, *A–C*, 328 (wild-type) and 1463 *(NK-1R*$^{-/-}$); Fig 3, *D*, 1463; Fig 3, *D, inset*, 314; and Fig 3, *E*, 787 (wild-type) and 246 (TRPV1-$Cre^{+/-}DTA^{+/-}$).

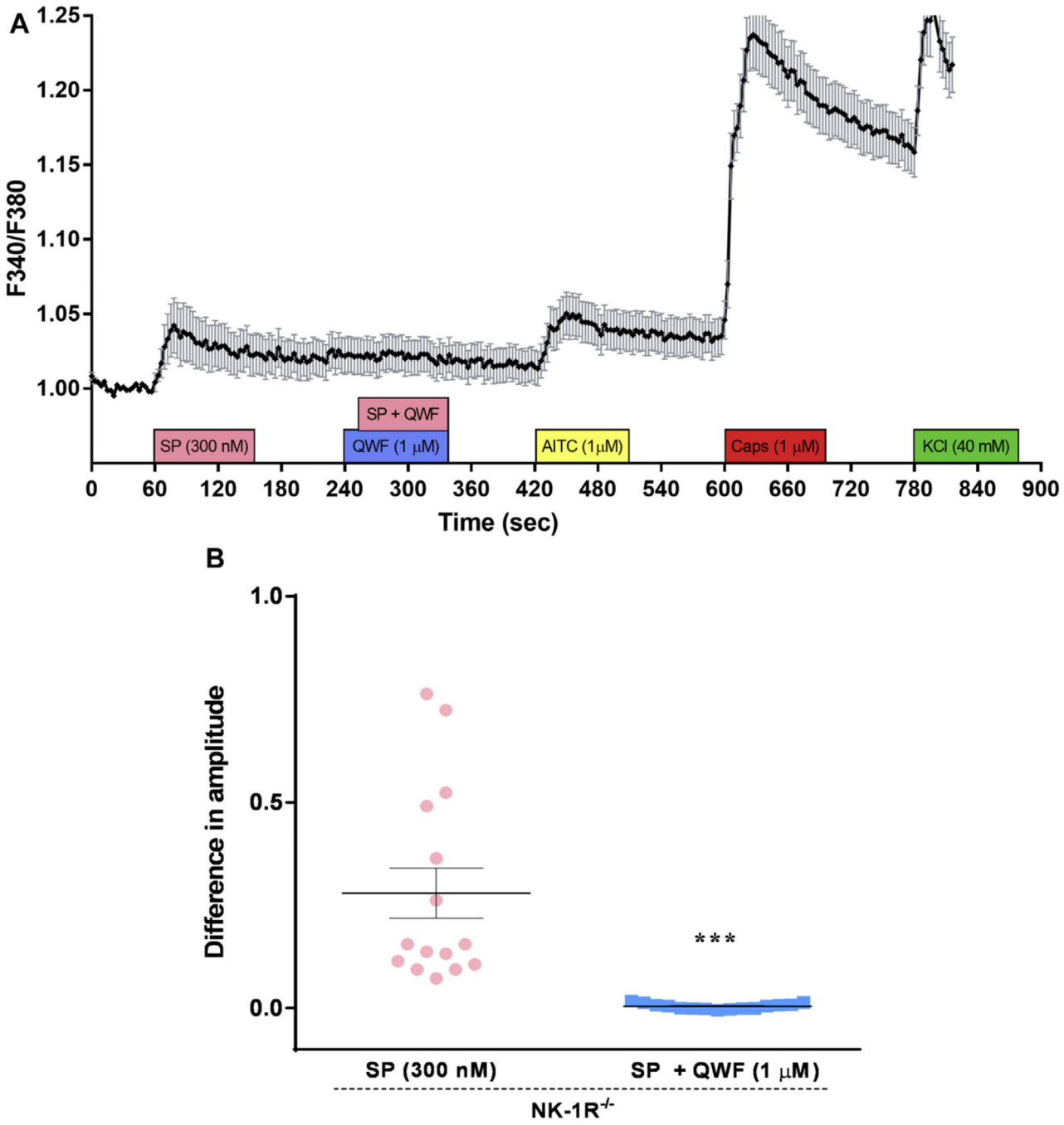

FIG 4.

Effect of QWF on SP-induced Ca^{2+} flux in cultured DRG neurons from *NK-1R*$^{-/-}$ mice. **A,** QWF (1 μmol/L) blocks SP-induced (300 nmol/L) Ca^{2+} flux in DRG neurons from *NK-1R*$^{-/-}$ mice. Cells blocked by QWF also respond to allyl isothiocyanate (1 μmol/L), capsaicin (1 μmol/L), and KCl (40 mmol/L). **B,** Amplitude of SP-induced Ca^{2+} flux in *NK-1R*$^{-/-}$ neurons before and after treatment with QWF (1 μmol/L). Data are expressed as means ± SEMs. The paired Student *t* test was used. Statistical difference from SP is

indicated as follows: ***$P < .001$. Total number of cells analyzed was as follows: Fig 4, *A*, 314; Fig 4, *B*, 492.