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Cl^- channel is required for CXCL-10-induced neuronal activation and itch response in a murine model of allergic contact dermatitis

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Author contributions:

L.Q. designed the experiments, conducted calcium imaging and electrophysiological experiments, and wrote the manuscript; K.F. and S.S. carried out the behavioral experiments and analyzed the behavioral data; R.H.L. designed the research, supervised the project and edited the manuscript.

Abstract

Persistent itch often accompanies allergic contact dermatitis (ACD), but the underlying mechanisms remain largely unexplored. We previously demonstrated that CXCL10/CXCR3 signaling activated a subpopulation of cutaneous primary sensory neurons and mediated itch response after contact hypersensitivity (CHS), a murine model of ACD, induced by squaric acid dibutylester. The purpose of this study was to determine the ionic mechanisms underlying CXCL10-induced neuronal activation and allergic itch. In whole-cell recordings, CXCL10 triggered a current in dorsal root ganglion (DRG) neurons innervating the area of CHS. This current was modulated by intracellular Cl^- and blocked by the general Cl^- channel inhibitors. Moreover, increasing Ca^{2+} buffering capacity reduced this current. In addition, blockade of Cl^- channels significantly suppressed CXCL10-induced Ca^{2+} response. In behavioral tests, injection of CXCL10 into CHS site exacerbated itch-related scratching behaviors. Moreover, the potentiating behavioral effects of CXCL10 were attenuated by either of two Cl^- channel blockers. Thus, we suggest that the Cl^- channel acts as a downstream target mediating the excitatory and pruritic behavioral effects of CXCL10. Cl^- channels may provide a promising therapeutic target for the treatment of allergic itch in which CXCL10/CXCR3 signaling may participate.

New & Noteworthy

The ionic mechanisms underlying CXCL10-induced neuronal activation and allergic itch are largely unexplored. This study revealed that CXCL10 evoked an ionic current mainly carried by Cl^- channels. We suggest that Cl^- channels are likely key molecular candidates responsible for the CXCL10-evoked neuronal activation and itch-like behaviors in a murine model of ACD induced by the antigen, SADBE. Cl^- channels may emerge as a promising drug target for the treatment of allergic itch in which CXCL10/CXCR3 signaling may participate.

Keywords: CXCR3; CXCL10; itch; pain; Cl^- channel; allergic contact dermatitis.

Introduction

Allergic contact dermatitis (ACD) is a common inflammatory skin disease initiated by T lymphocytes that are specific for an allergen (Grabbe and Schwarz 1998). Persistent itch (pruritus) and burning sensation are the major clinical sensory manifestations of ACD (Buddenkotte and Steinhoff 2010). Although the physiopathology of ACD is well studied, the pruritic mechanisms in ACD are largely unknown.

The C-X-C motif chemokine10 (CXCL10), also known as interferon-γ inducible protein 10 (IP-10), is exclusively expressed in ACD but not irritant contact dermatitis reactions (Enk and Katz 1992; Flier et al. 1999). CXCL10 is predominantly produced by epidermal cells in the challenged skin of CHS (Flier et al. 1999; Goebeler et al. 2001; Tokuriki et al. 2002) and modulates innate and adaptive immune responses by specifically attracting T cells and dendritic cells bearing its receptor CXCR3 to the site of allergen reaction (Dufour et al. 2002). In addition to immune cells, both CXCL10 and CXCR3 are detected in primary sensory neurons (Bhangoo et al. 2007) and have been implicated in the maintenance of a chronic pain state under inflammatory pain or neuropathic pain conditions (Bhangoo et al. 2007; Fu et al. 2010; Strong et al. 2012). Our recent study revealed that CXCL10/CXCR3 signaling was upregulated in dorsal root ganglion (DRG) neurons after CHS. Moreover, CXCL10 may exert its pruritic effects by directly exciting primary sensory neurons through CXCR3 (Qu et al. 2015). However, the ionic mechanisms underlying the excitatory and pruritic effects of CXCL10 are largely unexplored.

Chloride channels, including calcium-activated chloride channels (CaCCs), are present in primary sensory neurons and play an important role for the regulation of neuronal

excitability (Boudes et al. 2009; Hartzell et al. 2005). Moreover, in peripheral sensory neurons, the higher expression of sodium-potassium-chloride cotransporter increases $[Cl^-]_i$; therefore, the activation of CaCCs gives rise to the outward Cl^- flow and cell depolarization. (Kamaleddin 2017; Mao et al. 2012). Accordingly, Cl^- channels have been proposed to participate in somatosensory transduction. Indeed, anoctamin 1, one type of CaCCs, was identified to act as a heat sensor that mediates or amplifies thermal nociception (Cho et al. 2012). Certain pruritogens and algogens were shown to activate specific types of Cl^- channels to elicit acute pruritic and nociceptive responses, respectively (Cho et al. 2012; Liu et al. 2010). In addition, some types of Cl^- channels have been implicated in the maintenance of chronic neuropathic pain (Pineda-Farias et al. 2015). In murine microglia, Cl^- was identified as a key downstream transduction channel in CXCL10/CXCR3 signaling (Rappert et al. 2002). Therefore, our purpose was to investigate the potential role of Cl^- channels in mediating CXCL10-induced neuronal activation and allergic itch using a mouse model of CHS induced by a hapten, squaric acid dibutylester (SADBE).

Materials and Methods

Animals

C57BL/6 male mice used in the study were 2 to 3 months of age and weighed 20-30 g. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Yale University School of Medicine and were consistent with the guidelines provided by the National Institute of Health and the International Association for the Study of Pain.

Model of allergic contact dermatitis

Allergic contact dermatitis (ACD) or contact hypersensitivity (CHS) was elicited by using the contact sensitizer squaric acid dibutylester (SADBE; Sigma, St. Louis, MO), as described previously (Fu et al. 2014; Qu et al. 2014). Mice were sensitized with 1% SADBE in acetone (25 μl) topically applied to the shaved abdomen once daily for three consecutive days. Five days later, mice were challenged with a topical application of 1% SADBE (25 μl) onto the right cheek (for behavioral testing) for one day or, to the hairy skin of foot and the calf of hind leg (for electrophysiology and calcium imaging) once a day for two consecutive days. Separate groups of mice were challenged with the acetone alone and served as controls.

Retrograde labeling of cutaneous sensory neurons

Rationale for using neurons from DRGs rather than trigeminal ganglia (TG). We chose to study neurons from the DRG rather than TG because lumbar ganglia were used in our previous studies of the role of CXCL10/CXCR3 signaling in the mouse. We found that SADBE challenge to the skin of mouse cheek (cheek model) and calf (calf model) each induced analogous spontaneous itch- and pain-like behaviors directed to the skin of CHS (Qu et al. 2014). Moreover, CXCL10/CXCR3 signaling was involved in allergic itch associated with CHS in both cheek and calf models (Qu et al. 2015). Thus, it is likely that CHS caused the similar biological changes of DRG and TG neurons. DRG neurons instead of TG neurons were chosen for in vitro experiments.

For in vitro studies, DRG cell bodies were identified as cutaneous and as having innervated the area of CHS (or vehicle treatment) by the presence of a retrogradely transported red fluorescent dye, Dil (Invitrogen). Dil (1.7 mg /ml in 1% DMSO) was injected subcutaneously (s.c.) at the SADBE application sites on the hairy skin of the calf

147 (two injections) and also dorsum of the foot (one injection) of one hind leg of mice (10 µl
148 per site) at least 1 week before the 1st challenge with SADBE or acetone vehicle.

149 **Cultures of dissociated DRG neurons**

150 At 24 h after the 2nd challenge, L3-L5 lumbar DRGs, ipsilateral to either the acetone-
151 or SADBE-treated skin, were harvested and placed in oxygenated complete saline
152 solution (CSS) for cleaning and then mincing. The CSS consisted of (in mM): 137 NaCl,
153 5.3 KCl, 1 $MgCl_2$, 3 $CaCl_2$, 25 Sorbitol, and 10 HEPES, adjusted to pH 7.2 with NaOH.
154 For 20 min the DRGs were digested with 0.35 U/ml of Liberase TM (Roche Diagnostics
155 Corp., Indianapolis, IN) and then for 15 min with 25 U/ml of Liberase TL (0.25 U/ml;
156 Roche Diagnostics Corp.) and papain (30 U/ml, Worthington Biochemical, Lakewood,
157 NJ) in CSS containing 0.5 mM EDTA at 37°C. The tissue was triturated with a fire-
158 polished Pasteur pipette. The DRG neurons were suspended in DMEM medium
159 containing 1 mg/ml trypsin inhibitor and 1 mg/ml bovine serum albumin (Sigma) and
160 then plated onto poly-D-lysine/laminin coated glass coverslips (BioCoat, BD Biosciences,
161 MA). The DMEM medium had equivalent amounts of DMEM and F12 (Gibco, Grand
162 Island, MD) with 10% fetal calf serum (Sigma) and 1% penicillin and streptomycin
163 (Invitrogen). The cells were maintained in 5% CO_2 at 37°C in a humidified incubator and
164 used within 16-24 h after plating.

165 **Calcium imaging**

166 Calcium imaging was performed on cultured mouse DRG neurons, as described (Qu et
167 al. 2011). Only small-diameter neurons (≤ 25 µm) were used that were labeled as
168 cutaneous by the presence of Dil and innervated the chemically treated areas. DRG
169 neurons were first loaded with 2 µM Fura 2-acetoxymethyl ester (Invitrogen) in the dark

for 45 min at 37°C and subsequently washed twice in a HEPES buffer containing (in mM): 145 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES (adjusted to pH 7.4 with NaOH). DRG neurons were alternatively excited at 340 nm and 380 nm using a Polychrome V Monochromator (TILL Photonics). Images were recorded at 2-s intervals at a room temperature of 20-22°C using a cooled CCD camera (Sensicam, PCO, Germany) that was controlled by a computer with Image Workbench 5.2 software (Indec Biosystems, CA). The ratio of 340 nm to 380 nm fluorescence intensity [$R_{(340/380)}$] within a certain region of interest was used as a relative measure of the intracellular concentration of calcium ($[Ca^{2+}]_i$). At the end of the experiment, the viability of the neurons was confirmed by an increase in $[Ca^{2+}]_i$ evoked by a 5-s application of 50 mM K^+. Cells were considered to be responsive to a chemical if an increase in $R_{340/380}$ was equal or greater than 15% above baseline (Wilson et al. 2011). Mouse recombinant CXCL10 (50 nM, R&D Systems), niflumic acid (NFA, 100 μM in 0.1% DMSO, Sigma), or 4,4'-Diisothiocyanato-2,2'-stilbenedisulfonic acid (DIDS, 100 μM in 0.1% DMSO, Sigma) was added to HEPES buffer. Capsaicin ("CAP"; 1 μM; 10 s) was applied at the end of recordings to identify CAP- sensitive nociceptors. All agents were then applied locally to the neuronal cell bodies through a micropipette with a tip diameter of 100-μm-diameter and connected to an 8-channel pressure-controlled drug application system (AutoMate Scientific, CA).

Electrophysiological recordings

Whole-cell recordings were made from small-diameter (≤25 μm), Dil-labeled DRG neurons – typically those that had been identified as responsive to CXCL10 using calcium imaging. Whole-cell voltage-clamp experiments were performed at room

temperature of 20-22°C by means of a Multiclamp 700A amplifier and pClamp 9 software (Molecular Device, Sunnyvale, CA), as described (Qu et al. 2012; Qu et al. 2011). Signals were sampled at either 10 kHz or 20 kHz and were filtered at 2 kHz. The patch pipettes were pulled from borosilicate glass capillaries with a P97 horizontal puller (Sutter Instruments, Novarto, CA). The patch pipettes, after filled with internal solution, had a resistance of 3–4 MΩ and their series resistance was routinely compensated at 60-80%. Only neurons with a resting membrane potential more negative than -40 mV were included in the study.

The DRG neurons were continuously perfused with HEPES buffer. The regular internal solution contained (in mM): K^+-gluconate 120, NMDG-Cl- 30, $MgCl_2 \cdot 6H_2O$ 2, HEPES 10, MgATP 2, $CaCl_2 \cdot 2H_2O$ 1, EGTA 11, with pH adjusted to 7.2 using Tris-base. The high $[Cl^-]_i$ internal solution contained (in mM): NMDG-Cl 140, K^+-gluconate 30, $MgCl_2 \cdot 6H_2O$ 2, HEPES 10, MgATP 2, $CaCl_2 \cdot 2H_2O$ 1, EGTA 11, adjusted to pH 7.2. In the low $[Cl^-]_i$ internal solution, NMDG-Cl was decreased from 140 mM to 4 mM (Cho et al. 2012). Accordingly, K^+-gluconate was increased from 30 mM to 136 mM. The internal solution with high Ca^{2+} buffering capacity was obtained by replacing 11mM EGTA with 10 mM BAPTA in high $[Cl^-]_i$ internal solution.

Behavioral testing

For the "cheek model", either CXCL10 (2 µg/10 µl in PBS; R&D Systems) or its vehicle alone (10 µl PBS) was injected i.d. into the right cheek 24 h after the 1st SADBE challenge (when the skin was inflamed but in less fragile condition than after the 2nd challenge). Behavioral responses were video recorded with a camcorder for 30 min starting after the injection. The video recording was played back offline in slow-motion

to assess the total number of site-directed scratching bouts with the hind paw and wiping with the forepaw for 30 min (Fu et al. 2014). In other tests, the effects of chloride channel inhibitors on CXCL10-induced behavioral responses were tested. Either DIDS (50 nM/site, 10 µl; Sigma), NFA (50 nM/site, 10 µl; Sigma), or its vehicle alone (10 µl; 0.1 M $NaHCO_3$ in PBS) was injected i.d. into the right cheek 1 h before the cheek injection of CXCL10. The dose of Cl^- channel blockers was chosen based on pilot tests and published dose-response findings (Liu et al. 2010). All behavioral tests were performed by the experimenters blinded to experimental conditions.

Statistical analysis

Data were presented as means ± SEM. Student's t-test was used to test the significance of differences between means between two groups. Comparisons for more than three groups were carried out using a one-way analysis of variance (ANOVA) followed by Bonferroni multiple-comparison corrections. Comparisons of proportions were made using Fisher's exact test. The probability criterion for significant differences was $p < 0.05$. The type of statistical tests used for each comparison was indicated in the figure legends.

Results

CXCL10 activated a Cl^- conductance in cutaneous DRG neurons from CHS mice

To determine the ionic mechanisms underlying the CXCL10-induced membrane depolarization, we performed whole-cell recordings on the cultured cutaneous DRG neurons from CHS mice before and after the application of CXCL10. Bath application of CXCL10 (50 nM) for 2 min induced an inward current (I_{CXCL10}) with a peak amplitude of

68.3 ± 8.7 pA (n = 9) when the DRG neurons were held at -60 mV (Fig. 1A). Since Cl^- channels have been associated with the activation of ionic currents by CXCL10 in murine microglia (Rappert et al. 2002), we next asked whether the I_{CXCL10} recorded in DRG neurons was mediated by a Cl^- channel. The directly measured normal $[Cl^-]_i$ in DRG neurons was more than 30 mM (Rocha-Gonzalez et al. 2008). Thus, we set the Cl^- concentration in control internal solution at 36 mM. When the concentration of Cl^- was increased from 36 mM to 146 mM in the internal solution, the peak of the I_{CXCL10} was significantly potentiated (Fig. 1B,D). In contrast, lowering the concentration of Cl^- from 36 mM to 10 mM in the internal solution dramatically reduced the I_{CXCL10} (Fig. 1C,D), suggesting that this current is likely mediated by a Cl^- channel. Since the peak amplitude of the I_{CXCL10} was larger under the high $[Cl^-]_i$ condition, the high $[Cl^-]_i$ (146 mM) internal solution was chosen throughout the following experiments in order to facilitate the recordings of this current.

To further determine the potential involvement of Cl^- channels, we examined the effects of DIDS and NFA, the broad-spectrum chloride channel antagonists (Malekova et al. 2007), on the I_{CXCL10}. Pretreatment with DIDS (100 μM) or NFA (100 μM) for 3 min almost abolished the I_{CXCL10} (Fig. 2A-C, E), indicating that the current induced by CXCL10 was likely due to the opening of the Cl^- channels.

The CXCL10-induced Cl^- current was modulated by intracellular calcium in DRG neurons

Since the $[Ca^{2+}]_i$ was increased after exposure to CXCL10 (Qu et al. 2015) and certain types of Cl- channels are activated by intracellular Ca^{2+} (Duran et al. 2010; Hartzell et al.

2005), we next test whether intracellular Ca^{2+} modulated Cl^- channels induced by CXCL10. When the intracellular Ca^{2+} buffering capacity was enhanced by replacement of EGTA in the internal solution with the fast Ca^{2+} chelator, BAPTA (10 mM), the peak of the I_{CXCL10} was significantly attenuated (Fig. 2D-E), suggesting that CXCL10-induced Cl^- current was sensitized or regulated by intracellular Ca^{2+}.

Cl^- channels contributed to CXCL10-induced neuronal activation in CHS mice

CXCL10 was shown to activate cutaneous DRG neurons from CHS mice (Qu et al. 2015). We next asked whether Cl^- channels were involved in CXCL10-indued neuronal activation. In the presence of vehicle (0.1% DMSO), 42.1% (40 of 95) of cutaneous DRG neurons from CHS mice responded to CXCL10. Of all CXCL10-responsive neurons in CHS mice, 47.5% (19 of 40) were capsaicin insensitive, consistent with our published findings (Qu et al. 2015). Pre-incubation with a non-selective chloride channel blocker, NFA (100 μM) for 3 min significantly reduced the percentage of CXCL10-respsonsive neurons in CHS mice (Fig. 3). Of all the remaining CXCL10-responsive cells, 42.9 % (9 of 21) were capsaicin insensitive. These findings suggested that Cl^- channels may be required for the excitatory effects of CXCL10 in primary sensory neurons.

Cl^- channel was involved in CXCL10-mediated itch-like behaviors in CHS mice

Our recent study showed that CXCL10 injection into the cheek enhanced itch-related scratching behaviors in CHS but not in naïve mice (Qu et al. 2015). CXCL10 did not evoke pain-like wiping behaviors either in CHS or in naïve mice (Qu et al. 2015). Because Cl^- channels were identified as mediating the excitatory neuronal effects of CXCL10 in vitro, we next tested whether the potentiating effect of CXCL10 on

scratching behavior in CHS mice was mediated through Cl^- channels using the cheek model. At 24 h after the 1st challenge, i.d. injection of CXCL10 (i.d, 2 μg/10 μl) into the cheek of CHS mice significantly increased the number of scratching bouts as compared to the injection of vehicle (PBS) (Fig. 4A). There were no significant differences in the number of site-directed pain-like wiping behaviors between CXCL10 and vehicle alone (data not shown). Local i.d. injection of either of the Cl^- channel blockers DIDS (50 nM/site, 10 μl; i.d.) or NFA (50 nM/site; 10 μl; i.d.), but not either of their vehicles (0.1 M $NaHCO_3$ in saline; 10 μl), significantly reduced CXCL10-evoked scratching response in CHS mice (Fig. 4B), indicating that Cl^- channel contributes to CXCL10-elicted pruritic responses in the settings of skin inflammation.

Discussion

In this study, we have demonstrated that CXCL10 evokes an ionic current mainly carried by Cl^- channels. We suggest that Cl^- channels are likely key molecular candidates responsible for the CXCL10-evoked neuronal activation and itch-like behaviors in a murine model of ACD induced by the antigen, SADBE.

Our previous study found that cutaneous primary sensory neurons innervating the CHS skin became more excitable (Qu et al. 2014). Moreover, our recent findings revealed that upregulated CXCL10/CXCR3 signaling within DRG may contribute to neuronal hyperexcitability in the context of skin inflammation (Qu et al. 2015). The present study provided direct evidence to support the hypothesis that the Cl^- channel might represent an ionic mechanism mediating CXCL10-induced membrane depolarization in DRG neurons under the CHS condition. In this study, we observed that an increase in $[Cl^-]_i$ potentiated

309 CXCL10-induced inward current whereas a reduction in $[Cl^-]_i$ nearly abolished it.
310 Furthermore, this current was inhibited by general Cl^- channel antagonists. The
311 contribution of Cl^- currents to CXCL10/CXCR3 signaling was also revealed in murine
312 microglia (Rappert et al. 2002). Peripheral sensory neurons in comparison to neurons in
313 the central nervous system have a greater activity of cation-Cl^- cotransporters and thus
314 normally maintain higher $[Cl^-]_i$ levels (30 - 50 mM) (Mao et al. 2012). In addition,
315 inflammatory mediators may cause further Cl^- accumulation in the sensory neurons under
316 inflammatory conditions (Funk et al. 2008). Therefore, the equilibrium potential for Cl^- is
317 normally far more positive (-22 to -35 mV) than the resting membrane potential in
318 primary sensory neurons (-60 -55 mV) (Mao et al. 2012; Rocha-Gonzalez et al. 2008).
319 Thus, the activation of Cl^- conductance is thought to lead to the membrane depolarization
320 and neuronal excitation in primary sensory neurons (Cho et al. 2012; Liu et al. 2010) . In
321 addition, our study showed that blockade of Cl^- channels reduced the CXCL10-evoked
322 Ca^{2+} response, suggesting that Cl^- channel-induced depolarization is likely pro-excitatory
323 in DRG neurons. Further studies are required to identify the molecular identity of
324 CXCL10-activated Cl^- channels.
325 Our recent data showed that CXCL10 evoked a Ca^{2+} influx from the extracellular
326 space in DRG neurons (Qu et al. 2015). In the present study, we found that the I_{CXCL10}
327 was modulated by intracellular Ca^{2+}. Thus, it is likely that members of CaCCs may
328 contribute to CXCL10-activated Cl^- currents. One hypothesis is that CaCCs is activated
329 secondary to CXCL10-induced Ca^{2+} increase, causing membrane depolarization and a
330 further Ca^{2+} influx from extracellular space. However, our findings do not seem to
331 support this possibility because CXCL10-evoked Ca^{2+} responses were completely

inhibited by the Cl^- channel antagonist NFA. We suggest that CXCL10 binds to neuronal CXCR3 and activates a Cl^- conductance, which results in membrane depolarization and subsequent activation of voltage-gated Ca^{2+} channels. The CXCL10-evoked increase in $[Ca^{2+}]_i$ is probably due to an influx of calcium through voltage-gated Ca^{2+} channels. The elevated $[Ca^{2+}]_i$ may in turn enhance the activity of the Cl^- channels. However, the cellular signaling whereby CXCR3 is coupled to Cl^- channels in DRG neurons remains to be explored.

The upregulated excitatory neuronal CXCL10/CXCR3 signaling has been implicated in the chronic pain state in animal models of inflammatory pain (Bhangoo et al. 2007). Recently, we discovered that CXCL10, which is a nonpruritogenic chemokine in native mice, became a potent pruritogen that evoked itch-like behavior in ACD (Qu et al. 2015). In present study, we found that Cl^- channel antagonists greatly inhibited the CXCL10-elicted itch behavior in the mice with CHS, suggesting a potential role of Cl^- channels for CXCL10-evoked itch under the condition of skin inflammation. Indeed, Cl^- channels have been involved in acute nociception and itch induced by several algogens and pruritogens, including bradykinin, capsaicin, endothelin 1, and histamine (Cho et al. 2012; Deba and Bessac 2015; Liu et al. 2010). Moreover, some types of Cl^- channels, including anoctamin1, are able to detect nociceptive thermal stimuli and possibly mediate thermal nociception (Cho et al. 2012). In addition, Cl^- channels have been implicated in the maintenance of a chronic state of inflammatory and neuropathic pain (Garcia et al. 2014; Pineda-Farias et al. 2015). However, the contribution of Cl^- channels to spontaneous itch associated with CHS awaits further investigation. Since CXCR3 are widely expressed in immune cells, we cannot rule out a possible role of such non-neuronal cells in the pruritic effect

of CXCL10 and the anti-pruritic effects of Cl^- channel blockers in addition to the role of the sensory neurons.

In conclusion, we have demonstrated, for the first time to our knowledge, that Cl^- channels mediate CXCL10-induced neuronal excitation and allergic itch under the CHS condition. We suggest that blocking Cl^- channels may represent a therapeutic approach to treat the sensory symptoms of inflammatory disease where CXCL10/CXCR3 axis may participate.

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Figure Legends

Figure 1. The CXCL10-induced currents in DRG neurons after CHS were associated with the activation of a chloride conductance. The neurons were held at the membrane potential of -60 mV. **A**-**C**, Representative traces of inward currents (I_{CXCL10}) induced by CXCL10 (50 nM; 2 min) recorded with the internal solution containing concentrations of Cl^- that were normal (36 mM) (**A**), high (146 mM) (B), or low (10 mM) (**C**). **D**, Increasing $[Cl^-]_i$ dramatically enhanced the amplitude of I_{CXCL10} whereas lowering $[Cl^-]_i$ significantly attenuated this current. *$p < 0.05$ and **$p < 0.01$ versus normal $[Cl^-]$, one-way ANOVA with a Bonferroni post test.

Figure 2. Effects of Cl^- channel antagonists and intracellular Ca^{2+} on CXCL10-induced currents in DRG neurons innervating CHS skin. **A**-**B**, Sample traces of the I_{CXCL10} recorded in absence (**A**) and in the presence of the Cl^- channel blockers DIDS (100 μM) (**B**) or NFA (100 μM) (**C**) applied to the bath or in the presence of 10 mM BAPTA in the pipette solution (**D**). The high $[Cl^-]_i$ pipette solution was used. **E**, Pretreatment with DIDS or NFA for 3 min significantly reduced the peak amplitude of I_{CXCL10}. Replacement of 11 mM EGTA with 10 mM BAPTA in the pipette solution almost abolished this inward current. The numbers of DRG neurons tested are in parentheses. *$p < 0.05$ versus control, one-way ANOVA with Bonferroni post test.

Figure 3. Effects of Cl^- channel blockade on CXCL10-evoked Ca^{2+} responses in cutaneous DRG neurons after CHS. **A-B**, Representative traces of a CXCL10-evoked Ca^{2+} response in the presence of vehicle (0.1% DMSO) (**A**) and the Cl^- blocker NFA (100

537 μM) in the vehicle (**B**). **C**, Pre-incubation with NFA for 3 min, in comparison with its
538 vehicle, significantly suppressed the percentage of CXCL10-responsive neurons.
539 Numbers of responsive neurons divided by total number tested responding and/or tested
540 are given in parentheses. Cap: capsaicin. *$p < 0.05$ versus vehicle, Fisher's exact test.
541
542 **Figure 4**. Effects of Cl^- channel blockade on CXCL10-mediated itch-like behavior in
543 CHS mice. The number of bouts of site-directed scratching with the hind limb was
544 quantified for 30 min immediately after the injection. (**A**) At 24 h after the 1st challenge
545 with SADBE-(CHS), i.d. injection of CXCL10 (2 μg/10 μl in PBS vehicle) into the
546 SADBE-challenged cheek significantly increased the site-directed itch-related scratching
547 in comparison with PBS vehicle alone (Veh1). The number of animals tested is in
548 parentheses. *$p < 0.05$ versus vehicle, unpaired t tests. (**B**)The CXCL10-evoked
549 scratching in the SADBE-challenged cheek was significantly attenuated by pre-injection,
550 1 h before, with the general Cl^- channel blockers – either DIDS (1 μg/10 μl; i.d.) or NFA
551 (1 μg/10 μl; i.d.) in comparison with prior i.d. injection of the vehicle (Veh2; 0.1 M
552 $NaHCO_3$ in PBS). The number of animals tested is in parentheses.*$p < 0.01$ versus
553 vehicle, one-way ANOVA with Bonferroni post test.
554

Fig. 1

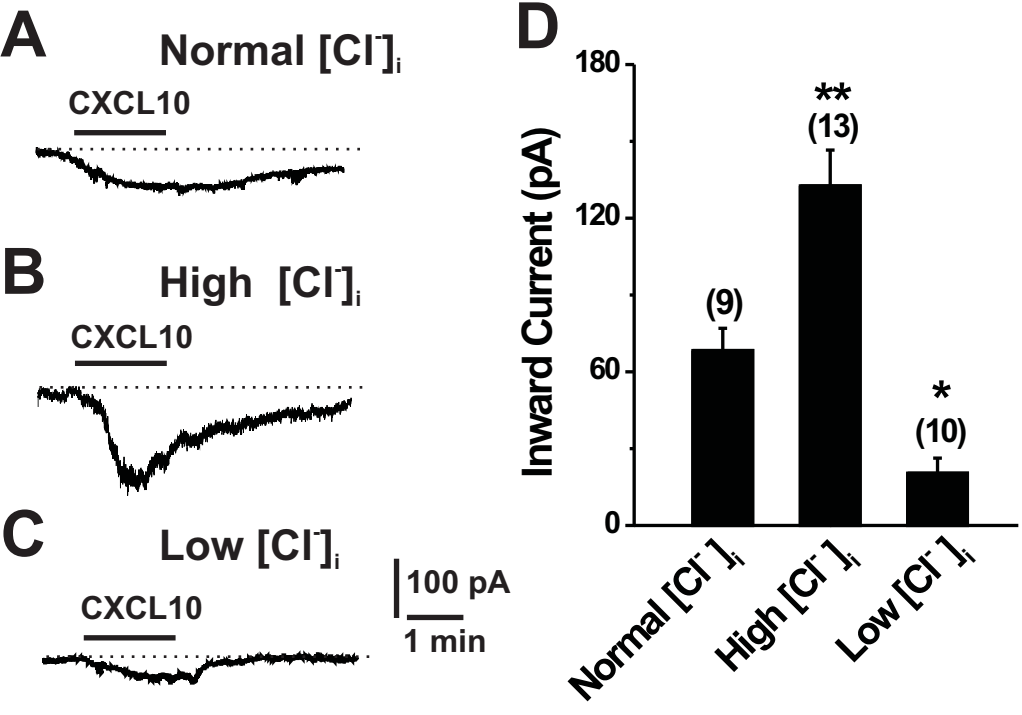
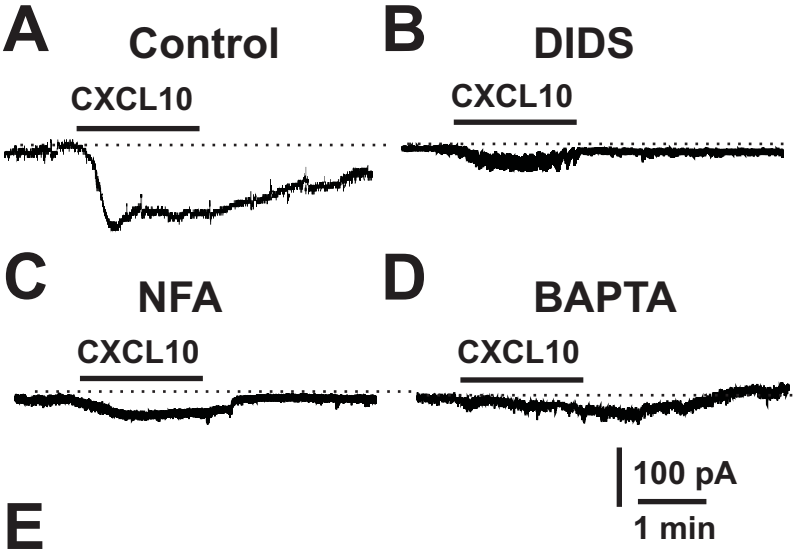

A
Normal [Cl-]i
CXCL10
B
High [Cl-]i
CXCL10
C
Low [Cl-]i
CXCL10
100 pA
1 min
D
Inward Current (pA)
180
120
60
0
**
(13)
(9)
*
(10)
Normal [Cl-]i
High [Cl-]i
Low [Cl-]i

Fig. 2


A
Control
CXCL10
B
DIDS
CXCL10
C
NFA
CXCL10
D
BAPTA
CXCL10
100 pA
1 min

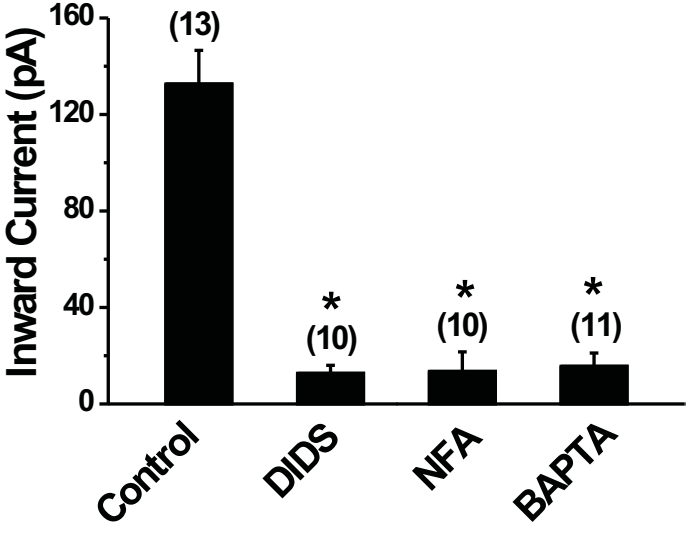

E
Inward Current (pA)
160
120
80
40
0
(13)
*
(10)
*
(10)
*
(11)
Control
DIDS
NFA
BAPTA

Fig. 3

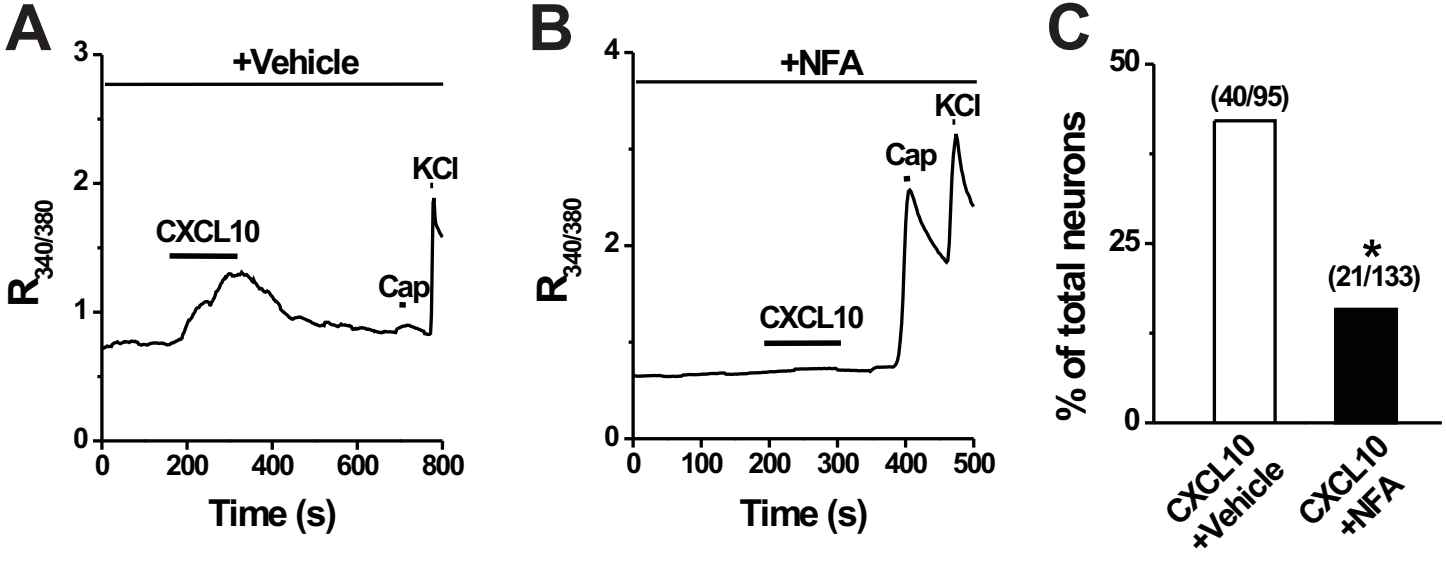
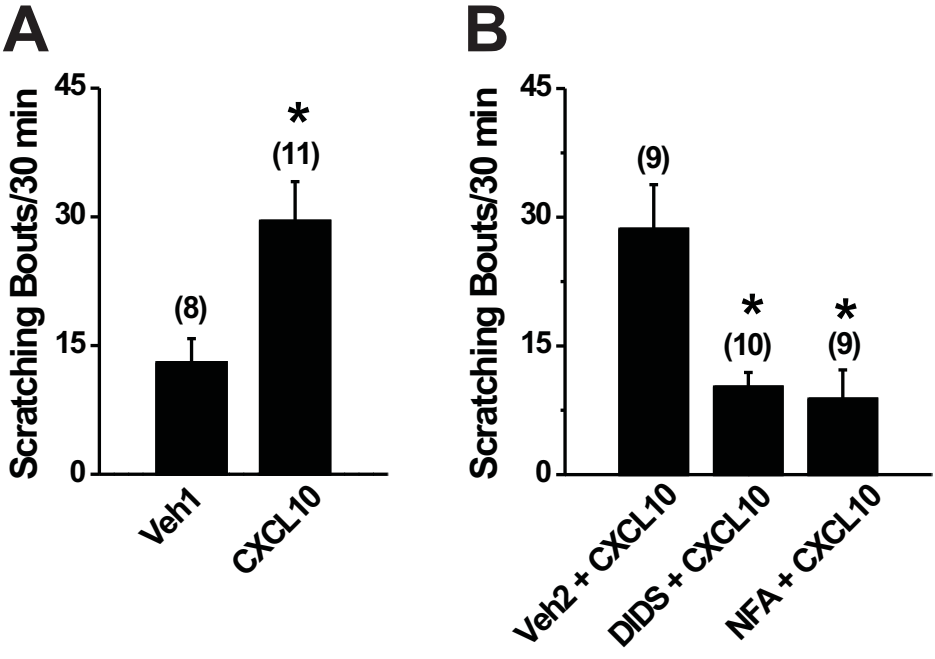

A
+Vehicle
CXCL10
Cap
KCl
$R_{340/380}$
Time (s)
B
+NFA
CXCL10
Cap
KCl
$R_{340/380}$
Time (s)
C
% of total neurons
(40/95)
(21/133)
*
CXCL10 +Vehicle
CXCL10 +NFA

Fig. 4


A
Scratching Bouts/30 min
45
30
15
0
(8)
*
(11)
Veh1
CXCL10
B
Scratching Bouts/30 min
45
30
15
0
(9)
*
(10)
*
(9)
Veh2 + CXCL10
DIDS + CXCL10
NFA + CXCL10