

# CCL2 and CXCL1 Trigger Calcitonin Gene-Related Peptide Release by Exciting Primary Nociceptive Neurons

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Chemokines are important mediators in immune responses and inflammatory processes. Calcitonin gene-related peptide (CGRP) is produced in dorsal root ganglion (DRG) neurons. In this study, CGRP radioimmunoassay was used to investigate whether the chemokines CCL2 and CXCL1 could trigger CGRP release from cultured DRG neurons of neonatal rats and, if so, which cellular signaling pathway was involved. The results showed that CCL2 and CXCL1 (~5–100 ng/ml) evoked CGRP release and intracellular calcium elevation in a pertussis toxin (PTX)-sensitive manner. The CGRP release by CCL2 and CXCL1 was significantly inhibited by EGTA,  $\omega$ -conotoxin GVIA (an N-type calcium channel blocker), thapsigargin, and ryanodine. Pretreatment of DRG neurons for 30 min with the inhibitors of phospholipase C (PLC) and protein kinase C (PKC) but not mitogen-activated protein kinases (MAPKs) significantly reduced CCL2- or CXCL1-induced CGRP release and intracellular calcium elevation. Intraplantar injection of CCL2 or CXCL1 produced hyperalgesia to thermal and mechanical stimulation in rats. These data suggest that CCL2 and CXCL1 can stimulate CGRP release and intracellular calcium elevation in DRG neurons. PLC-, PKC-, and calcium-induced calcium release from ryanodine-sensitive calcium stores signaling pathways are involved in CCL2- and CXCL1-induced CGRP release from primary nociceptive neurons, in which chemokines produce painful effects via direct actions on chemokine receptors expressed by nociceptive neurons. © 2005 Wiley-Liss, Inc.

**Key words:** CCL2; CXCL1; dorsal root ganglion; calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP), a 37-amino-acid peptide, has been widely identified in the central and peripheral neural systems. It is predominantly synthesized and stored in sensory neurons and can be released from both their central and their peripheral axons (Poyner, 1992). Considerable evidence indicates that the release of CGRP from sensory nerve terminals in peripheral tissues plays a key role in neurogenic

inflammation, whereas the release from terminals in the dorsal horn of the spinal cord modulates pain transmission (Oku et al., 1987; Holzer, 1988).

Chemokines are small, secreted proteins that stimulate the directional migration of leukocytes and mediate inflammation (Baggiolini et al., 1997). The chemokine receptor family is the largest family of G-protein-coupled receptors. Accordingly, the number of chemokines binding these receptors is large, with >50 chemokine peptides identified to date (Onuffer and Horuk, 2002). In addition to their potential roles in neuropathology, chemokines have recently been shown to trigger biochemical events such as stimulation of phosphoinositide-3 kinase (PI3 kinase) isoforms and activation of the ERKs/MAPK cascade (Meucci et al., 1998; Xia and Hyman, 2002; Limatola et al., 2002). Regulation of  $\text{Ca}^{2+}$  fluxes after chemokine receptor activation has also been described. In hippocampal neurons, CCL22, CCL5, CCL3, CX3CL1, and CXCL12 induce a rapid  $\text{Ca}^{2+}$  influx from the extracellular environment (Meucci et al., 1998). Nonetheless, stimulation of dorsal root ganglion (DRG) cells with CCL22 and CX3CL1 inhibits the  $\text{Ca}^{2+}$  influx, whereas CCL5 and CXCL12 do not (Oh et al., 2002).

Inflammation is associated commonly with states of heightened pain sensitivity. Recent reports suggest that chemokines may have additional roles to play in pain. CCL5 (ligands for CCR1, -3, -5, and -9), CXCL12 (ligand for CXCR4), and CCL22 (ligand for CCR4) induce pain when injected intradermally. Furthermore, DRG neurons express the chemokine receptors CX3CR1, CXCR4, CCR4, and CCR5, and CXCR4- and CCR4-positive neurons also express substance P, which has been implicated in nociception (Oh et al., 2001). Chemokine

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biology is further complicated by individual chemokines interacting with more than one receptor and chemokine receptors potentially binding more than one chemokine. The up-regulation of CCL2 production by the DRG neurons in a rat model of neuropathic pain is involved in the development of mechanical allodynia induced by nerve injury (Tanaka et al., 2004). We undertook the present study to determine whether CCL2 and CXCL1 could induce the release of CGRP and, if so, to explore the mediating mechanism and its possible implication.

## MATERIALS AND METHODS

### Preparation of DRG Neurons

The treatment of the laboratory animals and the experimental protocols adhered to the guidelines of the Health Science Center of Peking University and were approved by the Institutional Authority for Laboratory Animal Care. Cultures of DRG neurons from neonatal rats were prepared as described previously (Xing et al., 2001). Briefly, 5–7-day-old Sprague Dawley rats (220–250 g) were decapitated, and the DRGs were taken out rapidly, after which they were enzymatically digested with 0.125% collagenase I for 30 min. The precipitation was resuspended in 1.5 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.125% trypsin and incubated for 10 min at room temperature. After trypsin digestion, DRG neurons were washed twice, centrifuged, and then incubated with 0.4 mg/ml deoxyribonuclease (Dnase I), 0.55 mg/ml soybean trypsin inhibitor (1 mg inhibits 1.8 mg TRL), and 5 mM MgSO<sub>4</sub> with 10% fetal bovine serum for 30 min to stop the action of trypsin. After the enzyme solutions were removed, the ganglia were washed and centrifuged. The cell sediments were resuspended in DMEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate, 100 µM 5'-bromo-2-deoxyuridine, and 30 µM uridine. Individual cells were obtained from the ganglia by mechanical agitation with a fire-polished pipette. Cells were put into 24-well Costar culture dishes (16 mm diameter) precoated with poly-L-lysine (10 µg/ml). The cells were maintained in an atmosphere of 5% CO<sub>2</sub>/95% air at 37°C, and the growth medium was changed every 2 days. Cells were maintained in culture for 3–6 days prior to the studies.

### Release of CGRP From DRG Neurons

For release studies, the growth medium was aspirated from the culture wells; cells were washed with 1 ml DMEM, pH 7.40, and maintained at 37°C. Cells were incubated in 1 ml DMEM to measure the resting and basal release of CGRP and then incubated in DMEM containing various stimulators and reagents. After such exposure, the supernatants were removed from the culture wells, and the amount of CGRP-like immunoreactivity (CGRP-LI) was measured with use of a CGRP radioimmunoassay as previously described (Wang et al., 1992).

### Radioimmunoassay of CGRP

Briefly, the samples were reconstituted in 0.1 M phosphate buffer containing 0.1% bovine serum albumin (BSA),

0.01% NaN<sub>3</sub>, 50 mM NaCl, and 0.1% Triton X-100, pH 7.4. Standards of synthetic CGRP (rat amino acid sequence) ranging from 2.5 to 1,000 pg/assay tube or the sample, dissolved in a volume of 200 µl buffer, were incubated for 24 hr at 4°C with 100 µl anti-CGRP antibody (anti-human CGRP II antibody; Peninsula Laboratories, Belmont, CA) diluted in buffer. This antibody cross-reacts 100% with rat CGRP and shows <0.01% cross-reaction with human rat amylin and 0% cross-reaction with calcitonin, vasoactive intestinal polypeptide, substance P, and somatostatin (data from Peninsula Laboratories). The mixture was then incubated for an additional 24 hr at 4°C with 100 µl <sup>125</sup>I-labelled CGRP (10,000 cpm/tube; Amersham, Amersham, United Kingdom) in buffer. Free and bound fractions were separated by the addition of 100 µl goat anti-rabbit IgG (second antibody) and 100 µl normal rabbit serum for 2 hr at room temperature. An additional 0.5 ml of buffer were added, and the test tubes were centrifuged (3,000 rpm, 4°C) for 20 min. After the supernatant fractions were removed, the test tubes were analyzed for gamma radioactivity of <sup>125</sup>I remaining in the pellets. The IC<sub>50</sub> values for the CGRP radioimmunoassay were 30–40 pg/assay tube.

### Ca<sup>2+</sup> Imaging

Fluo-3/AM was used as the fluorescent Ca<sup>2+</sup> indicator. All measurements were made at room temperature. Preparation of DRG cells followed the methods described above. Neurons were plated in 35-mm culture dishes with glass bottoms (Costar, Cambridge, MA) precoated with poly-L-lysine (10 µg/ml) for culture and subsequent microscopy. Cells were loaded with 6 µM fluo-3/AM at 37°C for 30 min in HEPES-buffered HHSS (in mM: HEPES 20, NaCl 137, CaCl<sub>2</sub> 1.3, MgSO<sub>4</sub> 0.4, MgCl<sub>2</sub> 0.5, KCl 5.4, KH<sub>2</sub>PO<sub>4</sub> 0.4, Na<sub>2</sub>HPO<sub>4</sub> 0.3, NaHCO<sub>3</sub> 3.0, glucose 5.6, pH 7.4), followed by three washes and a 15-min incubation for further deesterification of fluo-3/AM before imaging. Then the cells were resuspended in 1 ml HHSS with different agents. Typically, time-lapse recording of Ca<sup>2+</sup> signals was a 50-sec control period before and a 3-min period after the application of different chemicals. The fluorescence signal was monitored at 488 nm wavelength and recorded with use of a confocal laser scanning microscope (Leica, Heidelberg, Germany).

### Pain Behavior Testing

To investigate whether CCL2 or CXCL1 (PeproTech EC) evokes hyperalgesia, rats received a unilateral intraplantar injection (100 or 500 ng in 5 µl to minimize the potential confound of inflammation and mechanical disruption) of vehicle, CCL2 (n = 8–10 per group), or CXCL1 (n = 7–9 per group). Thermal sensitivity was assessed by use of a hotplate (Hargreaves et al., 1988; Luo et al., 2004). The hotplate was set at 52.5°C and cutoff set at 40 sec. The latency until rats either licked their paws or jumped was recorded. Then, rats received an intradermal injection (100 or 500 ng in 5 µl) of vehicle, CCL2, or CXCL1 in the plantar surface of one hind paw. Thermal sensitivity was determined by measuring paw withdrawal latencies to heat stimulus at various time point (30, 60, 90, 120, 150, and 180 min) after injection.

Mechanical sensitivity to punctate tactile stimuli was determined with use of calibrated von Frey filaments by using the up-and-down paradigm as in our previous study (Chaplan et al., 1994; Sun et al., 2004). The response to mechanical threshold was determined 30, 60, 90, 120, 150, and 180 min after injection. All injections were made in a volume of 5  $\mu$ l to minimize the potential confound of inflammation and mechanical disruption.

### Chemicals and Drugs

CCL2 or CXCL1 were purchased from PeproTech EC. DMEM and FBS were obtained from Hyclone (Logan, UT). Deoxyriboxyribonuclease, collagenase, ryanodine, and fluo-3/AM were obtained from Sigma Chemical Co. (St. Louis, MO). Soybean trypsin inhibitor was purchased from Worthington Biochemical Corp. (Freehold, NJ). U73122, pertussis toxin (PTX), PD98059, SB202190, and SP600125 were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Calphostin C, RO-31-8220, and phorbol myristic acetate (PMA) were purchased from Research Biochemicals Inc. (Natick, MA).  $\omega$ -Conotoxin GVIA ( $\omega$ -CTX GVIA) was from Bachem Corp. (Torrance, CA). Thapsigargin was purchased from Biosciences Inc. (La Jolla, CA).

### Data Analysis

The data are expressed as mean  $\pm$  SEM. The data were analyzed by one-way ANOVA and further analyzed by the Student-Newman-Keuls test for multiple comparisons or unpaired Student *t*-test for means between two groups. *P* < 0.05 was considered significant.

## RESULTS

### CCL2 and CXCL1 Evoked CGRP Release and $[Ca^{2+}]_i$ Increase Via Gi/Go in DRG Neurons

First, we tested the response of CGRP release in DRG neurons to the administration of CCL2 and CXCL1. CCL2 and CXCL1 significantly enhanced

the release of CGRP from DRG neurons in a time-dependent fashion (Fig. 1A). As shown in Figure 1B, the release of CGRP treated with various concentrations of CCL2 (10–100 ng/ml) or CXCL1 (50–100 ng/ml) for 6 hr were significantly increased compared with control.

To explore the possibility that CCL2 and CXCL1 might evoke an increase in  $[Ca^{2+}]_i$  in DRG neurons,  $[Ca^{2+}]_i$  monitoring revealed that 10–100 ng/ml CCL2

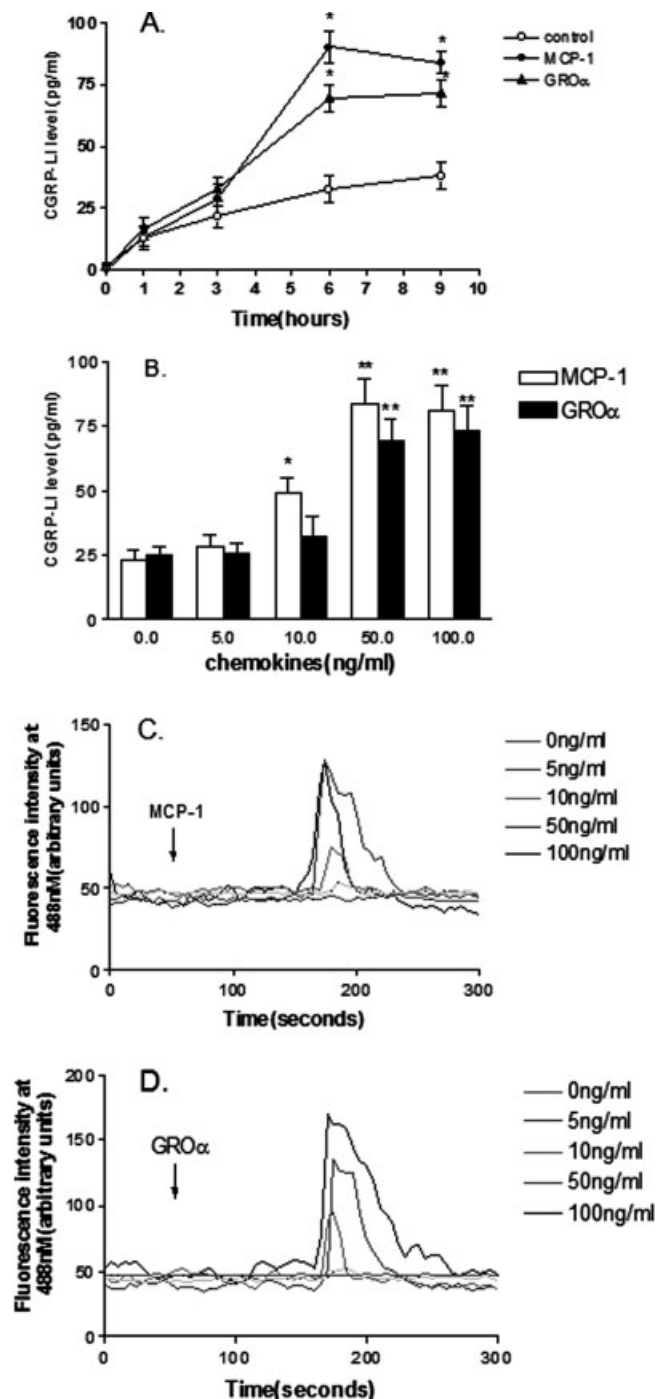


Fig. 1. Effect of CCL2 or CXCL1 on CGRP release and  $[Ca^{2+}]_i$  increase in DRG neurons. **A:** Time course of CCL2- or CXCL1-induced CGRP release. DRG neurons were incubated with vehicle control (open circles), 50 ng/ml CCL2 (solid circles) or 50 ng/ml CXCL1 (triangles) for the indicated periods. The levels of CGRP in the medium were measured at the indicated times after treatment. **B:** DRG neurons were incubated with CCL2 (open squares) or CXCL1 (solid squares) at the indicated concentrations for 6 hr, and the medium was then removed and analyzed for CGRP-LI levels. Data represent mean  $\pm$  SEM of at least five independent experiments. \**P* < 0.05, \*\**P* < 0.01 compared with control. **C,D:** Changes in  $[Ca^{2+}]_i$  level induced by various concentrations of CCL2 or CXCL1 in the fluo-3/AM-loaded DRG neurons. Each trace represents changes of fluorescence intensity over time in an individual neuron acquired every 5 sec (see Materials and Methods). CCL2 or CXCL1 was applied to neurons at the time indicated by the arrow. The  $[Ca^{2+}]_i$  levels rapidly returned to basal levels in the presence of CCL2 or CXCL1. A total of 58 of 104 neurons and 67 of 152 neurons showed  $[Ca^{2+}]_i$  increase with 50 and 100 ng/ml CCL2 stimulation, respectively. A total of 62 of 123 neurons and 51 of 97 neurons responded to 50 ng/ml CXCL1 and 100 ng/ml CXCL1, respectively.

evoked a rapid increase in level of  $[Ca^{2+}]_i$  beginning at 125 sec after treatment, which rapidly decreased to the basal level 200 sec later (Fig. 1C). CXCL1 (10–100 ng/ml) increased  $[Ca^{2+}]_i$  in DRG neurons in a manner similar to that for CCL2, reaching the maximum within 130 sec after treatment (Fig. 1D).

To assess the specific effect of CCL2 or CXCL1 on the production of CGRP in DRG cells, CCL2 (50 ng/ml) and anti-CCL2 (500 ng/ml) or CXCL1 (50 ng/ml) and anti-CXCL1 (500 ng/ml) were coincubated for 2 hr at 37°C before being added to cell cultures. Normal rabbit IgG was used as the negative control. The stimulatory effect of CCL2 or CXCL1 on CGRP release was abrogated by anti-CCL2 or anti-CXCL1 but not rabbit IgG (Fig. 2A,B).

Chemokine receptors have long been known to be coupled with G proteins sensitive to bacterial toxin (Ward and Westwick, 1998). To test which G protein was involved in the CCL2- or CXCL1-induced CGRP release and  $[Ca^{2+}]_i$  elevation, we incubated DRG neurons with PTX (500 ng/ml, overnight), an inhibitor of Gi/Go protein. PTX had no effect on basal responses but completely abrogated the CCL2- or CXCL1-induced CGRP release and  $[Ca^{2+}]_i$  increase (Fig. 3A,B), which indicates the involvement of the Gi/Go protein.

#### Role of PLC-PKC-MAPK Pathway in the CCL2- or CXCL1-Induced CGRP Release and $[Ca^{2+}]_i$ Elevation

To examine whether PLC is involved in CCL2- or CXCL1-induced CGRP release and  $[Ca^{2+}]_i$  elevation, we preincubated cells with 5–10  $\mu$ M U73122, the inhibitor of PLC, for 20 min at 37°C and then stimulated them with 50 ng/ml CCL2 or CXCL1. It was found that the amount of CGRP released from DRG neurons was reduced, after treatment with 10  $\mu$ M U73122 (Fig. 4A). U73122 was preliminarily tested to verify its inability to evoke CGRP release in DRG neurons. Likewise, the inhibitory effects observed in the 50 ng/ml CXCL1-stimulated neurons preincubated with U73122 (5–10  $\mu$ M) were also observed (Fig. 4B). The attenuation of CCL2- or CXCL1-stimulated CGRP release shows the involvement of PLC signaling in these responses.

Moreover, pretreatment of DRG cells for 30 min with calphostin C (500 nM) or RO-31-8220 (100 nM), the two PKC inhibitors, significantly blocked 50 ng/ml CCL2- or CXCL1-induced CGRP release. In contrast, neurons incubated with 0.1  $\mu$ M PMA, an activator of PKC, for 20 min significantly increased the CGRP release (Fig. 5A,B).

The effect of PKC inhibitors on CCL2- or CXCL1-induced cytoplasmic calcium mobilization was also investigated. As shown in Figure 5C,D, when PKC activity was largely (or completely) inhibited by 500 nM calphostin C, the increases in  $[Ca^{2+}]_i$  resulting from CCL2 or CXCL1 were completely abrogated by cal-

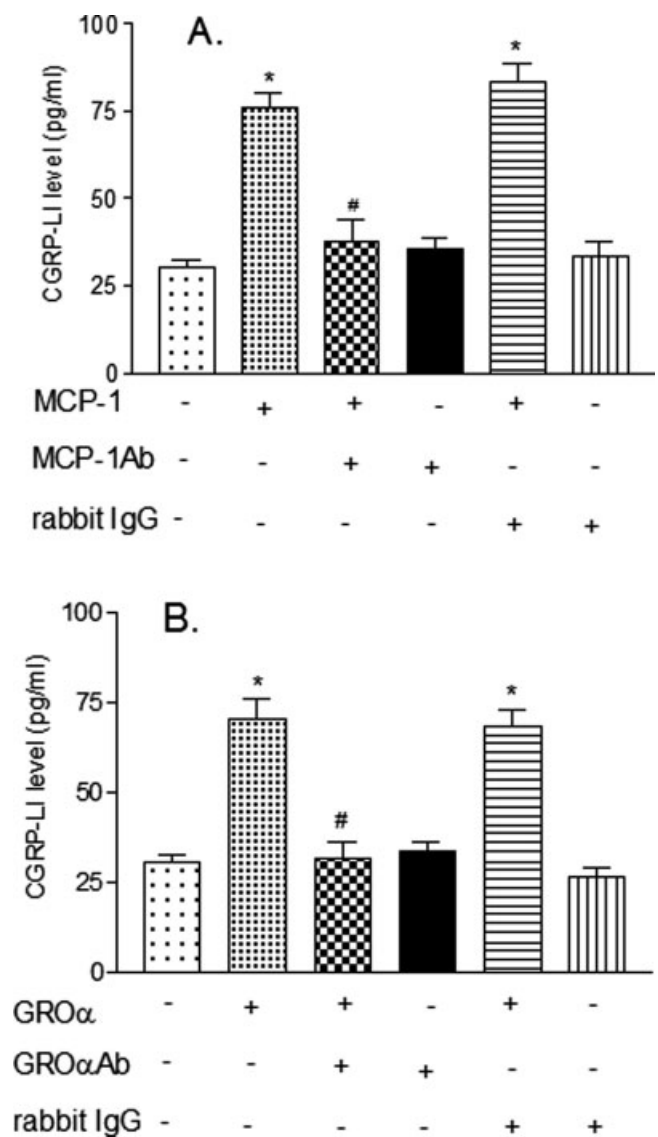


Fig. 2. Effect of anti-CCL2 or anti-CXCL1 on CCL2- or CXCL1-induced CGRP release in DRG neurons. DRG neurons were incubated with 50 ng/ml CCL2 (A), 50 ng/ml CXCL1 (B), and/or 500 ng/ml anti-CCL2 (A), 500 ng/ml anti-CXCL1 (B), or normal rabbit IgG for 6 hr. The supernatant was then removed and analyzed for CGRP-LI levels. Data are mean  $\pm$  SEM of duplicate cultures, representative of three independent experiments. \* $P$  < 0.05 compared with control; # $P$  < 0.05 compared with chemokine alone.

phostin C (500 nM), which suggests the involvement of PKC in CCL2- or CXCL1-evoked intracellular calcium elevation.

The activation of ERK1/2, members of the MAPK family, by various chemokine receptors has been reported. ERK1 and -2 have been reported to have important roles in the chemoattractant-mediated signaling as downstream of PTX-sensitive G-protein-coupled receptors (Hii et al., 1999; Bonacchi et al., 2001). We investigated the possible coupling between the MAPK pathways and CCL2- or

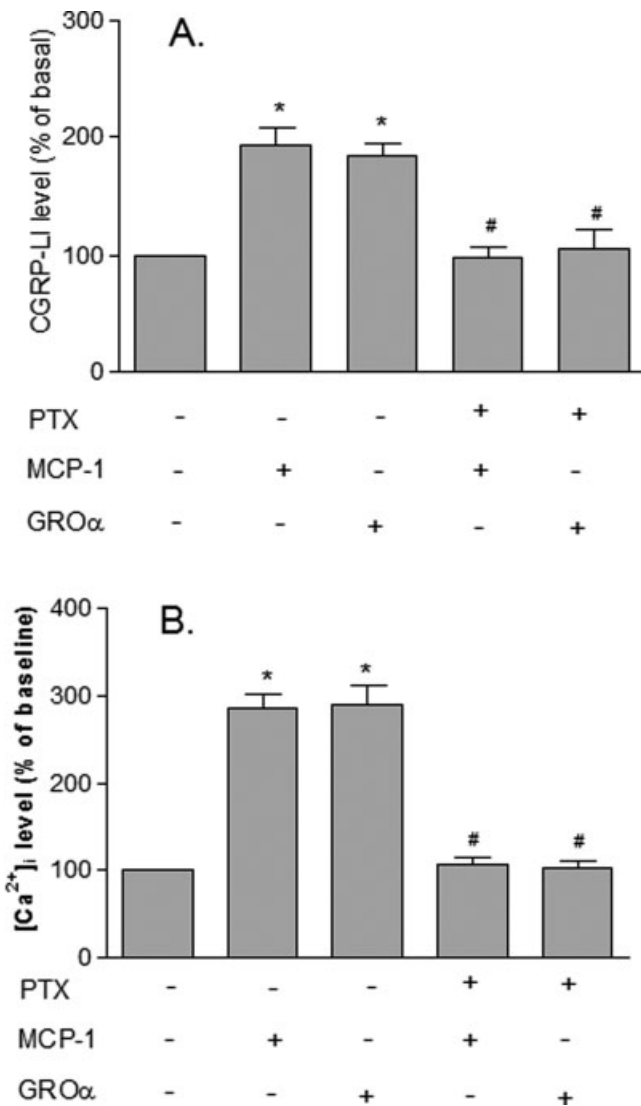


Fig. 3. Pertussis toxin (PTX) abrogated the CCL2- or CXCL1-induced CGRP release and  $[Ca^{2+}]_i$  elevation. **A:** DRG neurons, untreated or preincubated 24 hr with 500 ng/ml PTX, were exposed to 50 ng/ml CCL2 or 50 ng/ml CXCL1 for 6 hr. The medium was then removed and analyzed for CGRP-LI levels. **B:** CCL2 or CXCL1 evoked  $[Ca^{2+}]_i$  increase in a PTX-sensitive manner. Bar graph summarizes the blockade of  $[Ca^{2+}]_i$  elevation resulting from CCL2 or CXCL1 by PTX. DRG neurons were cultured in the presence of 500 ng/ml PTX for 24 hr prior to the addition of 50 ng/ml CCL2 or CXCL1 for 5 min. Neurons were monitored for  $[Ca^{2+}]_i$  level. Changes in  $[Ca^{2+}]_i$  level after application of chemokines were quantified by normalizing the fluorescence intensity at an optimal time point to that of the control period and presented as the percentage of basal level. Data are presented as mean  $\pm$  SEM from many neurons ( $n = 48-56$ ). \* $P < 0.05$  compared with control; # $P < 0.05$  compared with CCL2 or CXCL1 alone.

CXCL1-induced release of CGRP in DRG neurons. For this purpose, DRG neurons were treated with PD98059 (20  $\mu$ M), a specific ERK inhibitor; SB202190 (8  $\mu$ M), a p38 inhibitor; or SP600125 (400 nM), a JNK inhibitor.

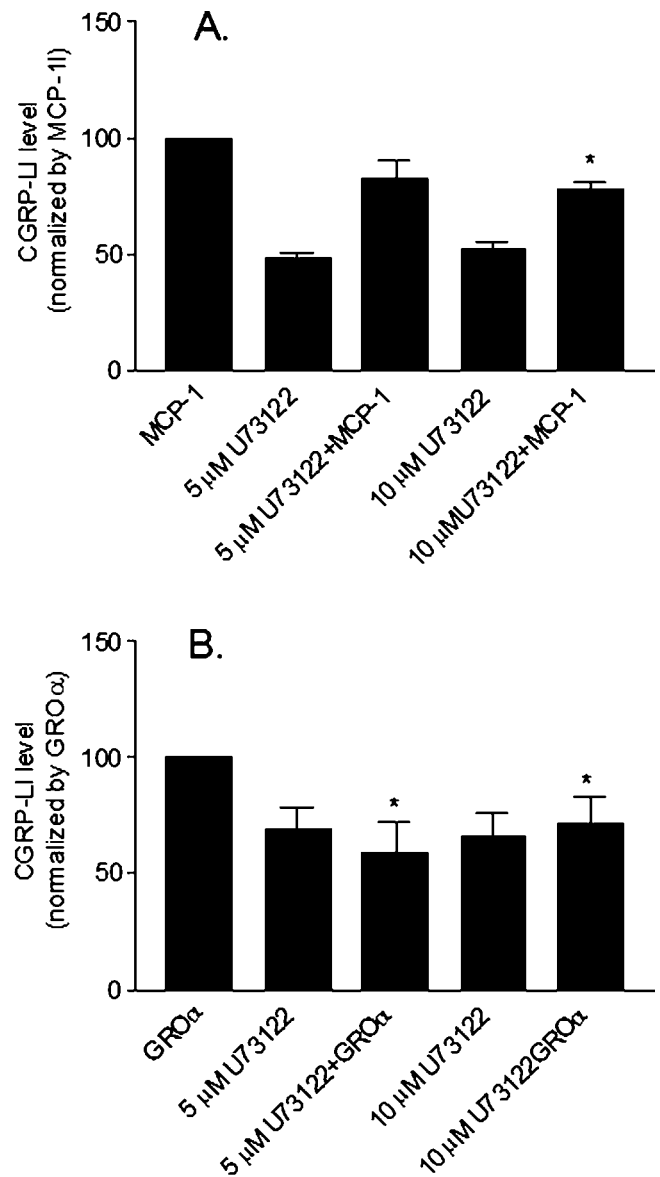


Fig. 4. Effects of U73122 on CCL2- or CXCL1-induced CGRP release in DRG cells. DRG cells were preincubated with U73122 at 5  $\mu$ M and 10  $\mu$ M for 30 min and then stimulated with 50 ng/ml CCL2 (**A**) or 50 ng/ml CXCL1 (**B**) for 6 hr. The medium was then removed and analyzed for CGRP-LI levels. Data are mean  $\pm$  SEM of five independent experiments. \* $P < 0.05$  compared with CCL2 or CXCL1.

The levels of CGRP released and intracellular calcium elevated because of CCL2 or CXCL1 were unaffected by these inhibitors of MAPKs, even at higher concentrations (data not shown). All these inhibitors were preliminarily tested to verify their inability to evoke CGRP release in DRG neurons. Thus, the MAPKs pathway did not mediate the effects of CCL2- or CXCL1-induced CGRP release and intracellular calcium increase in DRG neurons.

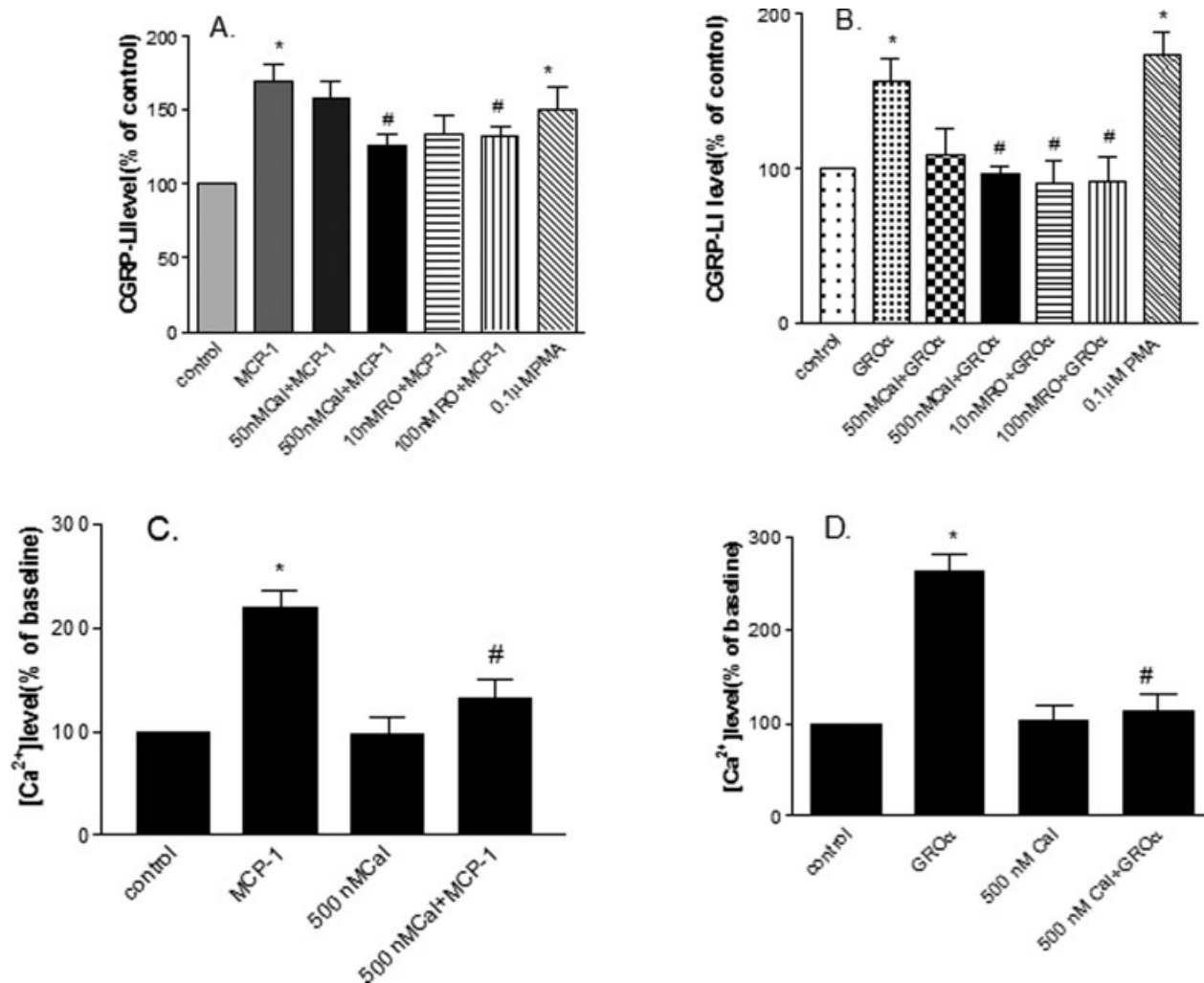


Fig. 5. Role of PKC pathway in CCL2- or CXCL1-evoked CGRP release and  $[Ca^{2+}]_i$  elevation in DRG cells. Cells were pretreated with calphostin C (Cal; 50 nM or 500 nM) or RO-31-8220 (RO; 10 nM or 100 nM) for 30 min before incubation with 50 ng/ml CCL2 (A) or 50 ng/ml CXCL1 (B) for 6 hr. Cells were also incubated with 0.1  $\mu$ M PMA for 20 min. The medium was then removed and analyzed for CGRP-LI levels. Data represent mean  $\pm$  SEM of at least five independent experiments. C,D: Cells were pretreated with calphostin C (Cal; 500 nM) for 30 min before incubation with 50 ng/ml CCL2 (C) or 50 ng/ml CXCL1 (D) for 5 min.

The fluo-3/AM loaded neurons (in HHSS containing 1.3 mM  $Ca^{2+}$ , pH 7.4) were monitored for  $[Ca^{2+}]_i$  level. Changes in  $[Ca^{2+}]_i$  level after application of CCL2 were quantified by normalizing the fluorescence intensity at the optimal time point to that of the control period and presented as the percentage of baseline. Data are presented as mean  $\pm$  SEM from many neurons. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. chemokines alone.

### Role of Extracellular $Ca^{2+}$ and N-Type Calcium Channels in CCL2- or CXCL1-Induced CGRP Release in DRG Neurons

Given that intracellular  $Ca^{2+}$  elevation in DRG neurons was a prominent feature of the response to CCL2 or CXCL1, we hypothesized that the changes in  $[Ca^{2+}]_i$  might be involved in CCL2- or CXCL1-induced CGRP release. CCL2- or CXCL1-stimulated CGRP release was measured in  $Ca^{2+}$ -containing HHSS and  $Ca^{2+}$ -free HHSS plus 2 mM EGTA, an effective chelator for extracellular  $Ca^{2+}$ . In chelated  $Ca^{2+}$ , 50 ng/ml CCL2- or CXCL1-stimulated CGRP release was inhibited

markedly (Fig. 6A,B). Thus, extracellular  $Ca^{2+}$  is important for CCL2- or CXCL1-stimulated CGRP release in DRG neurons.

To determine whether CCL2 or CXCL1 enhances CGRP release by stimulating voltage-activated calcium channels, the specific blocker of N-type calcium channel  $\omega$ -CTX GVIA (1  $\mu$ M) was added to cultures of DRG cells prior to the addition of CCL2 or CXCL1.  $\omega$ -CTX inhibited CCL2- or CXCL1-evoked CGRP release (Fig. 6C,D), which suggests that such release depends on calcium influx via an N-type calcium channel from the extracellular space.

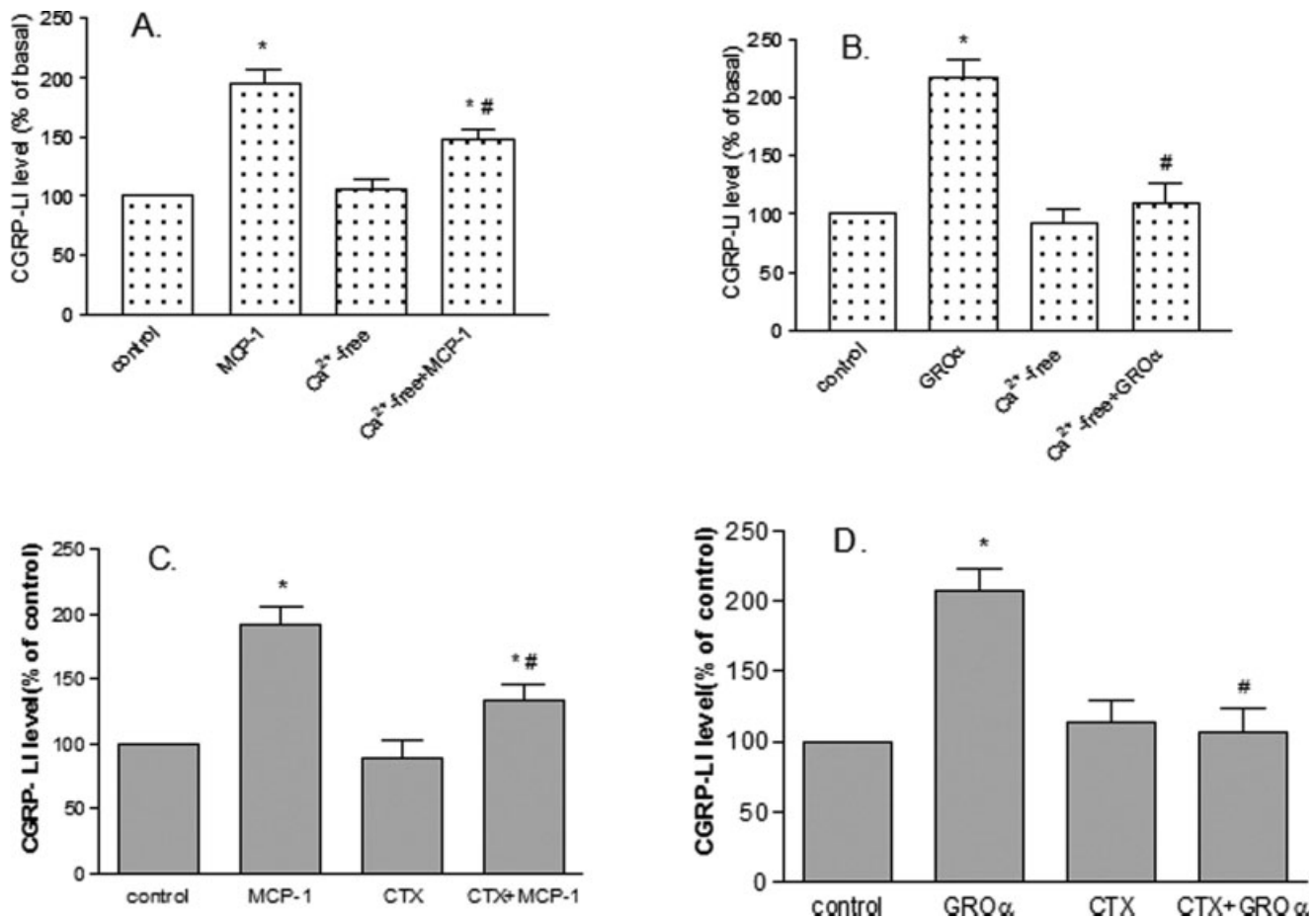


Fig. 6. Role of extracellular calcium and N-type calcium channel in CCL2- or CXCL1-stimulated CGRP release in DRG neurons. The neurons were incubated in HHSS containing 1.3 mM Ca<sup>2+</sup> and Ca<sup>2+</sup>-free HHSS containing 2 mM EGTA for 30 min, respectively, and then stimulated with 50 ng/ml CCL2 (A) or 50 ng/ml CXCL1 (B) for 6 hr. The medium was then removed and analyzed for CGRP-LI levels. Data are mean  $\pm$  SEM of triplicate cultures, repre-

sented three independent experiments. \* $P$  < 0.05 vs. control; # $P$  < 0.05 vs. CCL2 or CXCL1 alone. C,D: Cells were pretreated with 1  $\mu$ M  $\omega$ -conotoxin GVIA (CTX) for 30 min before incubation with 50 ng/ml CCL2 (C) or 50 ng/ml CXCL1 (D) for 6 hr. The medium was then removed and analyzed for CGRP-LI levels. Data represent mean  $\pm$  SEM of at least five independent experiments. \* $P$  < 0.05 vs. control. # $P$  < 0.05 vs. CCL2 or CXCL1 alone.

### Role of Internal Ca<sup>2+</sup> Stores in CCL2- or CXCL1-Induced CGRP Release

An influx of extracellular Ca<sup>2+</sup> can stimulate a further Ca<sup>2+</sup> release from internal stores, which might also be involved in the action of CCL2 or CXCL1. Thapsigargin, a highly potent inhibitor of the internal membrane Ca<sup>2+</sup>-ATPase, was used to empty internal Ca<sup>2+</sup> stores. Pretreatment with thapsigargin (10  $\mu$ M) efficiently suppressed CCL2- or CXCL1-induced CGRP release (Fig. 7A), whereas thapsigargin per se did not induce any change in CGRP release. These data indicate that the release of Ca<sup>2+</sup> from internal stores is involved in CCL2- or CXCL1-evoked CGRP release from DRG neurons.

Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR) from internal stores is regulated by the activation of a calcium channel known as the "ryanodine receptor" (RyR; Herzi and Mcdermott, 1991). Pretreatment with high concentra-

tions of ryanodine (10  $\mu$ M), a blocker of ryanodine receptor, substantially reduced the CCL2- or CXCL1-evoked CGRP release (Fig. 7B,C), which suggests that ryanodine-sensitive internal Ca<sup>2+</sup> stores are also involved in the CCL2- or CXCL1-induced CGRP release from DRG cells.

### CCL2- and CXCL1-Induced Allodynia

The ability of CCL2 and CXCL1 to release CGRP through exciting nociceptive neurons implies that they might produce nociception. In the hotplate test (Fig. 8A), the latency at the tested temperature (52.5°C) was shortened after 120 min in rats receiving intraplantar injections of CCL2 (500 ng, but not 100 ng;  $n$  = 8–10) compared with the vehicle group. Likewise, the groups of rats receiving a unilateral intraplantar injection of 500 ng CXCL1 ( $n$  = 7–9) also displayed a shortened licking

latency, appearing after 120 min and with 100 ng CXCL1 at 180 min (Fig. 8B).

Responsiveness to punctuate mechanical stimuli was determined with use of von Frey filaments. At a dose of 500 ng CCL2 ( $n = 8-9$ ), the decrease of 50% mechanical threshold to withdraw the hind paw in response to punctuate mechanical stimuli was observed,

and maximal nociceptive response was detected 90 min after the injection (Fig. 8C). Intraplantar injection of 500 ng of CXCL1 ( $n = 7-9$ ) also produced withdrawal responses after 30 min. This effect was maximal until 120 min after injection, and its magnitude was greater than that produced by CCL2 (Fig. 8D).

## DISCUSSION

Our results demonstrate for the first time that the chemokines CCL2 and CXCL1 not only promote CGRP release but also induce intracellular calcium elevation in a time- and concentration-dependent manner in cultured rat DRG cells. We also compared the signaling of CCL2-induced CGRP release with that of CXCL1. CCL2 and CXCL1 share signaling via the PTX-sensitive G protein/PLC/PKC, but not the MAPKs pathway. The PKC pathway promotes calcium influx via an N-type calcium channel, which induces calcium release from ryanodine-sensitive calcium stores that are responsible for the rapid release of CGRP resulting from CCL2 or CXCL1 stimulation. Intraplantar injection of CCL2 or CXCL1 might produce hyperalgesia to heat and mechanical stimuli by, at least in part, releasing sensory nerve neurotransmitter CGRP.

Chemokines constitute a superfamily of small proteins (8–14 kDa) that are instrumental for trafficking leukocytes in normal immunosurveillance as well as coordinating the infiltration of inflammatory cells under pathological conditions. Chemokines and their receptors form an elaborate signaling system. Currently, approximately 50 human chemokines have been described, and these chemokines interact with 18 different chemokine receptors (Murphy et al., 2000; Zlotnik and Yoshie, 2000). Chemokines are classified by their structure on the basis of the number and spacing of conserved cysteine motifs in the  $\text{NH}_2$  terminus. Thus, four groups, the C, CC, CXC, and CX3C families, have been distinguished. The classification of the chemokine receptors parallels the four subgroups: XCR, CCR, CXCR, and CX3CR. Most of these chemokine receptors recognize more than one chemokine.

It is becoming clear that neurons express a wide variety of chemokine receptors. Indeed, chemokines and

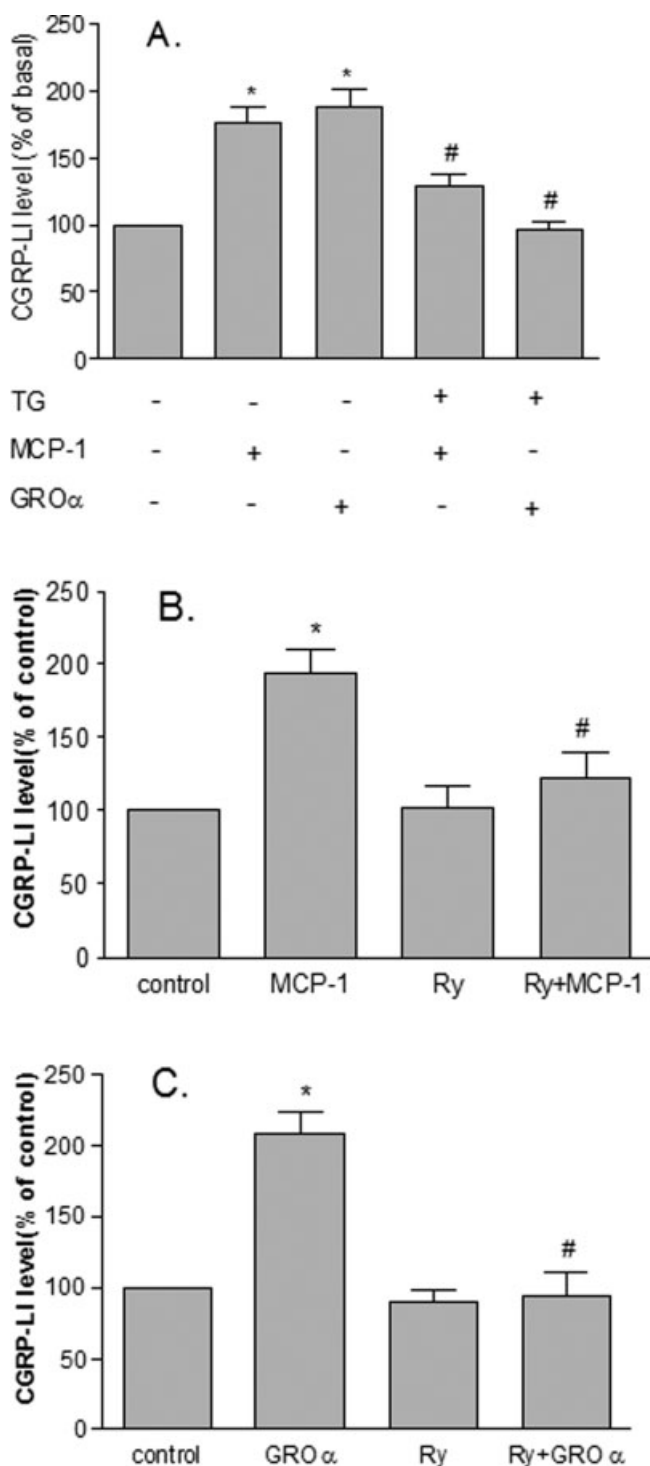


Fig. 7. Role of thapsigargin-sensitive  $\text{Ca}^{2+}$  stores and ryanodine receptor in CCL2- and CXCL1-induced CGRP release from DRG neurons. **A:** Cells were pretreated with 10  $\mu\text{M}$  thapsigargin (TG) for 30 min, and the supernatant was removed and analyzed for CGRP-LI levels by radioimmunoassay. TG per se did not induce CGRP release. Then, the cells were treated with 10  $\mu\text{M}$  TG and 50 ng/ml CCL2 or 50 ng/ml CXCL1 concomitantly for 6 hr. The medium was then removed and analyzed for CGRP-LI levels by CGRP radioimmunoassay. **B,C:** Cells were pretreated with 10  $\mu\text{M}$  ryanodine (Ry) for 30 min, and the supernatant was removed. Then, the cells were treated with 50 ng/ml CCL2 (B) or 50 ng/ml CXCL1 (C) and ryanodine (10  $\mu\text{M}$ ) concomitantly for 6 hr. The supernatants were removed and analyzed for CGRP-LI levels. Data represent mean  $\pm$  SEM of at least five independent experiments. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. chemokine alone.



their receptors are expressed by all of the major cell types throughout the nervous system (Miller and Meucci, 1999). The neuronal expression of many chemokine receptors suggests a direct chemokine effect on neurons. Thus, chemokines might play a variety of roles in the nervous and the immune systems. In support of this possibility, chemokines have been reported to produce a number of short-term effects on synaptic transmission and long-term effects on neuronal survival (Miller and Meucci, 1999; Zheng et al., 1999; Limatola

et al., 2002). Here, we provide evidence that the ligands CCL2 and CXCL1 can be a potent trigger for the release of CGRP and the increase of intracellular calcium in DRG neurons.

Signaling mechanisms by chemokine receptors have been studied mainly in the hematopoietic system. Although not completely understood, their signaling pathways appear to bear resemblance to the pathway of the classic G-protein-coupled receptors (Bacon, 1997). Direct effects of other chemokines on neurons have also been reported: the activation of neuronal ERK1/2 pathway by CXCR3 ligands CXCL9 and CXCL10 (Xia et al., 2000); the modulation of neurotransmitter release in the rat cerebellum by the CXCR2 ligand CXCL1 (Ragozzino et al., 1998); the increase of intracellular  $Ca^{2+}$  in rat hippocampal neurons by CXCL8, CXCL12 (a CXCR4 ligand), CX3CL1 (a CX3CR1 ligand), and CCL22 (a CCR4 ligand); and a neuroprotective effect by CCL22, CCL5, CXCL12, or soluble CX3CL1. Both CX3CL1 and CCL22 produce a time-dependent activation of ERK1/2, whereas no activation of c-JUN NH<sub>2</sub>-terminal protein kinase (JNK) stress-activated protein kinase or p38 MAPK was evident in hippocampal neurons (Meucci et al., 1998). CXCL1/KC can be a potent trigger for the ERK1/2 and PI3 kinase pathways in mouse primary cortical neurons (Xia and Hyman, 2002). However, our current data do not suggest the involvement of MAPK pathways in the CCL2 and CXCL1/KC stimulation of CGRP release in DRG cells of neonatal rats.

Our study shows that CCL2- and CXCL1-induced CGRP release and  $[Ca^{2+}]_i$  elevation are inhibited by two PKC inhibitors. In addition, PMA, which is used to stimulate PKC activity, can significantly trigger the release of CGRP. These results indicate that the PKC signaling pathway is involved in CCL2- and CXCL1-evoked CGRP release. It has been reported that activation of PKC increases the release of CGRP and substance P from the rat sensory neurons and spinal cord slices. The inhibition of PKC can significantly reduce the release of sensory neuropeptides (Barber and Vasko, 1996; Frayer et al., 1999; Hou and Wang, 2001), and its

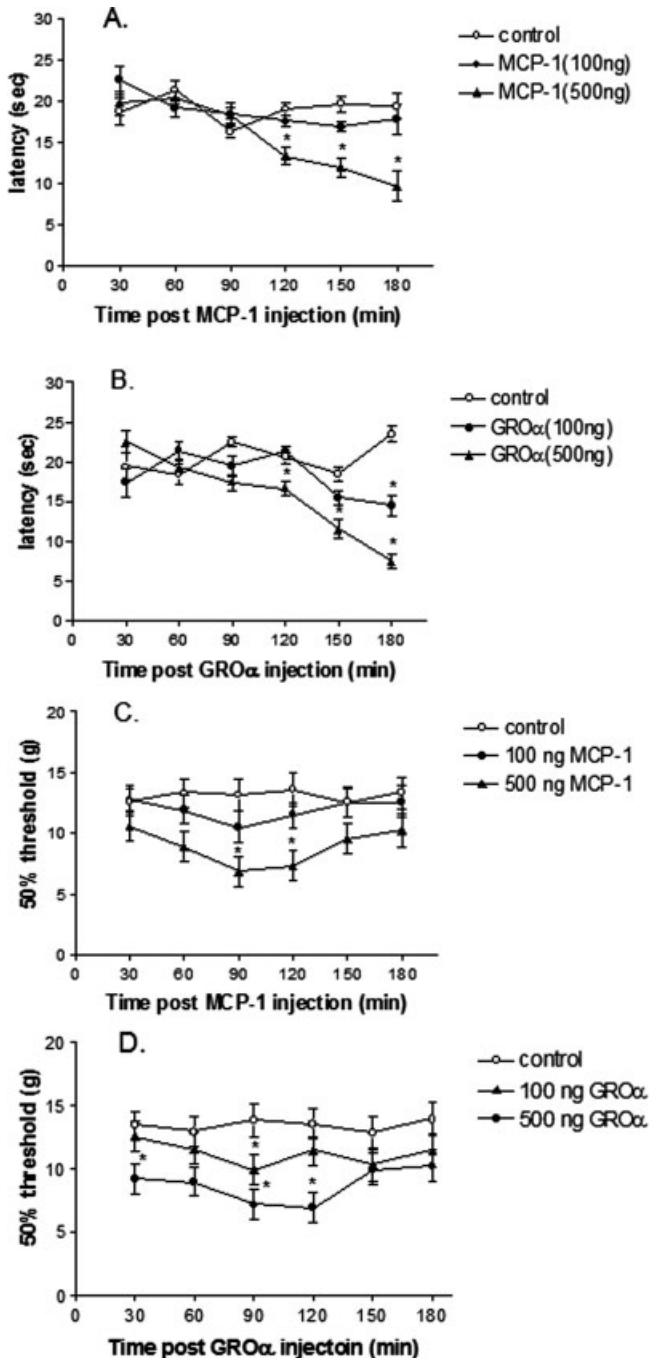


Fig. 8. Paw withdrawal latencies and mechanical hypersensitivity to noxious stimuli after injection of CCL2 or CXCL1. **A:** The intradermal administration of 500 ng CCL2 (triangles), but not the vehicle control (open circles) and 100 ng CCL2 (solid circles), reduced the time to licking in response to thermal stimuli. **B:** The intradermal administration of 100 ng CXCL1 (solid circles) or 500 ng CXCL1 (triangles) reduced the time to licking in response to thermal stimuli compared with the vehicle control (open circles). **C:** The intradermal administration of 500 ng CCL2 (triangles), but not the vehicle control (open circles) and 100 ng CCL2 (solid circles), reduced the 50% mechanical threshold to withdraw the hind paw in response to punctate mechanical stimuli. **D:** The intradermal administration of 100 ng CXCL1 (solid circles) or 500 ng CXCL1 (triangles) reduced the 50% mechanical threshold to withdraw the hind paw in response to punctate mechanical stimuli. Data are mean  $\pm$  SEM from seven to ten rats. \* $P < 0.05$  vs. the corresponding time point in vehicle-treated rats.

activation induces and facilitates noxious heat-induced CGRP release (Kessler et al., 1999).

It is widely accepted that calcium plays a key role in neurotransmitter release. Calcium acts as a universal second messenger, coupling the external stimuli with a variety of intracellular processes. Calcium influx through neuronal voltage-activated calcium channels mediates a range of cytoplasmic responses, such as neurotransmitter release and activation of calcium-dependent enzymes (Smith and Augustine, 1988; McCormack et al., 1990). The N-type calcium channel is the first distinct type of Ca channel demonstrated to be involved in transmitter release largely on the basis of blockage with the specific toxin  $\omega$ -CTX-GVIA (Kerr and Yoshikami, 1984; Miller, 1987). Fura-2-based  $\text{Ca}^{2+}$  imaging showed that numerous chemokines, including CXCL12, CCL5, and CX3CL1, affect neuronal  $\text{Ca}^{2+}$  signaling (Meucci et al., 1998). Our findings show that CCL2 and CXCL1 can increase intracellular calcium level blocked by the PKC inhibitor, which suggests that the CCL2- and CXCL1-induced intracellular calcium increase depends on calcium influx via PKC pathway. Our present work also demonstrates that  $\omega$ -CTX-GVIA, a specific blocker of the N-type calcium channel, can inhibit CCL2- and CXCL1-induced CGRP release. These data indicate that PKC may elevate cytoplasmic calcium through an N-type calcium channel in DRG neurons.

Furthermore,  $\text{Ca}^{2+}$  released from intracellular stores probably contributes to the CGRP release. This is likely, because thapsigargin, which depletes intracellular stores, significantly suppresses the CCL2- and CXCL1-induced CGRP release. The influx of extracellular  $\text{Ca}^{2+}$  can stimulate a further  $\text{Ca}^{2+}$  release from ryanodine receptor  $\text{Ca}^{2+}$ -release channels, a mechanism called " $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release" (CICR; Herzi and Mcdermott, 1991). Our group recently reported that lipopolysaccharide induced CGRP release from rat DRG neurons by the calcium-induced calcium release mechanism (Qin et al., 2004). The present study shows that ryanodine pretreatment to abrogate  $\text{Ca}^{2+}$  release suppresses CCL2- and CXCL1-induced CGRP release. The activation of PLC generates  $\text{IP}_3$ , which can activate  $\text{IP}_3$  receptors in calcium-containing stores, thereby releasing  $\text{Ca}^{2+}$ . Most chemokines share the ability to activate G-protein-sensitive PLC isoforms, resulting in  $\text{IP}_3$  generation and elevation of intracellular calcium (Sozzani et al., 1993; Kuang et al., 1996). Insofar as pretreatment with the PLC inhibitor U73122 reduces CCL2- and CXCL1-induced CGRP release,  $\text{IP}_3$  receptors might be also involved in the action of CCL2 and CXCL1.

The phenomenon of inflammation-induced hyperalgesia has been recognized for a long time. A repertoire of cellular mediators (e.g., bradykinin, PG, and nerve growth factor) has been shown to enhance the sensitivity of nociceptive neurons to noxious stimuli. It has been shown that CCR2 $^{-/-}$  mice do not develop the mechanical allodynia typically associated with neuropathy, and the chemokine-mediated recruitment and activation of macrophages and microglia in skin and nerve tissue might contribute to

both inflammatory and neuropathic pain states (Abbadie et al., 2003). The current behavioral observation shows the potencies of CCL2 and CXCL1 in inducing hyperalgesia. Taken together with the actions of these agents on DRG neurons, it is reasonable to assume that the allodynia produced by the chemokines arises from actions exerted at the peripheral terminals of the small-diameter nociceptors. However, this observation does not exclude an action of these agents at nonneuronal sites in the hind paw. Recent work (Milligan et al., 2000) indicates that chemokines can produce allodynia and hyperalgesia via actions involving microglia in the spinal cord. However, the small volume and amounts administered into the hind paw make it unlikely that sufficient quantities of the chemokines accessed this site. Thus, these data provide the initial evidence for a local site of action on CGRP-containing sensory nerve endings. Oprea and Kress (2000) reported that cytokines, interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), significantly increased the amount of released CGRP during heat (47°C) stimulation from rat skin in vitro, where the CGRP release from rat skin might result from an acute action of IL-1 $\beta$  and TNF $\alpha$ . In comparison with the present study, the responses induced by IL-1 $\beta$  (20 ng) or TNF $\alpha$  (50 ng) seem more potent than that of CCL2 or CXCL1 during the exposure to heat (47°C) stimulation of rat skin in vitro. In inflammation, both proalgesic (e.g. cytokines, chemokines, growth factors, and bradykinin) as well as analgesic mediators (e.g., opioid peptides, antiinflammatory cytokines, endocannabinoids, and somatostatin) are generated (Watkins and Maier, 2002; Rittner et al., 2003). The best-characterized endogenous analgesic system is the opioid peptides (Stein et al., 2003). It has been shown that chemokines, CCL3 and CCL5, are capable of desensitizing  $\mu$ -opioid receptors (MOR) on peripheral sensory neurons. The desensitization of MORs provides a means of suppressing their analgesic effects and as a result promotes pain signals centrally as well as in the peripheral nervous system (Zhang et al., 2004). Activation of proinflammatory chemokine receptors down-regulates the analgesic functions of opioid receptors and, therefore, enhances the perception of pain at inflammatory sites (Szabo et al., 2002). The number of CGRP-like immunoreactive (-LI) neurons in DRG cultures following repeated treatment with different concentrations of various opioid receptor agonists is significantly increased (Belanger et al., 2002). The data from the present study suggest that CCL2 and CXCL1 may inhibit analgesic effects and as a result promote pain signals centrally as well as in the peripheral nervous system.

In summary, the present study shows, for the first time, that CCL2 and CXCL1 can trigger CGRP release and  $[\text{Ca}^{2+}]_i$  increase in a time- and concentration-dependent manner in rat DRG cells. PTX-sensitive G-protein/PLC/PKC signaling pathways and CICR are involved in the CCL2- and CXCL1-induced CGRP release events in these cells. CCL2 and CXCL1 may participate in the occurrence of nociception via, at least in part, neurotransmitter CGRP release from sensory nerve terminals.

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