

# MCP-1 Enhances Excitability of Nociceptive Neurons in Chronically Compressed Dorsal Root Ganglia

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**Sun, J. H., B. Yang, D. F. Donnelly, C. Ma, and R. H. LaMotte.** MCP-1 enhances excitability of nociceptive neurons in chronically compressed dorsal root ganglia. *J Neurophysiol* 96: 2189–2199, 2006. First published June 14, 2006; doi:10.1152/jn.00222.2006. Previous experimental results from our laboratory demonstrated that monocyte chemoattractant protein-1 (MCP-1) depolarizes or increases the excitability of nociceptive neurons in the intact dorsal root ganglion (DRG) after a chronic compression of the DRG (CCD), an injury that upregulates neuronal expression of both MCP-1 and mRNA for its receptor CCR2. We presently explore the ionic mechanisms underlying the excitatory effects of MCP-1. MCP-1 (100 nM) was applied, after CCD, to acutely dissociated small DRG neurons with nociceptive properties. Under current clamp, the proportion of neurons depolarized was similar to that previously observed for CCD-treated neurons in the intact ganglion, although the magnitude of depolarization was greater. MCP-1 induced a decrease in rheobase by  $44 \pm 10\%$  and some cells became spontaneously active at resting potential. Action potential width at a voltage equal to 10% of the peak height was increased from  $4.94 \pm 0.23$  to  $5.90 \pm 0.47$  ms. In voltage clamp, MCP-1 induced an inward current in 27 of 50 neurons held at  $-60$  mV, which increased with concentration over the range of 3 to 300 nM ( $EC_{50} = 45$  nM). The MCP-1-induced current was not voltage dependent and had an estimated reversal potential of  $-27$  mV. In addition, MCP-1 inhibited a voltage-dependent, noninactivating outward current, presumably a delayed rectifier type  $K^+$  conductance. We conclude that MCP-1 enhances excitability in CCD neurons by, at least, two mechanisms: 1) activation of a nonvoltage-dependent depolarizing current with characteristics similar to a nonselective cation conductance and 2) inhibition of a voltage-dependent outward current.

## INTRODUCTION

A chronic compression of the dorsal root ganglion (CCD) is an animal model of neuropathic pain because it increases the excitability of the sensory neurons in the compressed ganglion and is accompanied by spontaneous pain behavior, cutaneous hyperalgesia, and tactile allodynia (Hu and Xing 1998; Song et al. 1999). The neuronal hyperexcitability of dorsal root ganglion (DRG) neurons might contribute to the neuropathic pain after CCD, although underlying mechanisms are still under investigation (Ma and LaMotte 2005; Tan et al. 2006; Yao et al. 2003).

Cytokines are thought to contribute to neuropathic pain behavior in CCD rats. For example, a local perfusion of the DRG with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in rats induces cutaneous hyperalgesia to mechanical stimuli by eliciting a PKA-dependent excitation in sensory neurons. Conversely, the

blocking of activity of certain inflammatory cytokines in the DRG partially relieves the CCD-induced hyperalgesia (Homma et al. 2002; Zhang et al. 2002).

Chemokines are small chemotactic cytokines of about 10 kDa secreted in damaged tissue, for example, from neurons (White et al. 2005) or nonneuronal cells such as activated glia and leukocytes (Feng 2000; Murdoch and Finn 2000; Rossi and Zlotnik 2000; Subang and Richardson 2001; Taskinen and Roytta 2000; White et al. 2005). Monocyte chemoattractant protein-1 (MCP-1)/CCL2, a  $\beta$  or C-C family member of chemokines, is a potent chemoattractant and activator for monocytes (Gangur et al. 2002; Gu et al. 1999). CCR2, initially identified in leukocytes, is a major receptor for MCP-1. Several lines of evidence have revealed a neuronally expressed MCP-1 and CCR2 (Banisadr et al. 2002, 2005; Coughlan et al. 2000; Meng et al. 1999; Tran and Miller 2003), suggesting that MCP-1/CCR2 signaling plays important physiological and pathophysiological roles in the CNS. Some chemokines may directly activate DRG neurons (Oh et al. 2001) and recent studies suggest that MCP-1/CCL2 and its C-C chemokine receptor, CCR2, might play a key role in neuropathic pain (Abbadie et al. 2003; Tanaka et al. 2004; White et al. 2005).

We recently demonstrated that MCP-1 depolarizes nociceptive neurons in the intact DRG after CCD and that this is correlated with an upregulation of the neuronal expression of both MCP-1 and mRNA for its receptor, CCR2 (Sun et al. 2005; White et al. 2005). Accordingly, we speculated that MCP-1/CCR2 signaling might contribute to allodynia and hyperalgesia in CCD rats. Because CCR2 receptor staining was also present in most of the nonneuronal cells around DRG neurons in the injured DRG, it raised a question as to whether MCP-1 directly excited neurons or whether it acted on nonneuronal cells to release factors such as TNF $\alpha$  or interleukin-1 $\beta$  (Ohtori et al. 2004; Sekiguchi et al. 2004) that excited the DRG neurons. The present study was designed to test the hypothesis that MCP-1 exerts direct effects on the excitability of the cell bodies of nociceptive DRG neurons after CCD. For this purpose, MCP-1 was applied to DRG neurons, acutely dissociated from their axons and satellite glia. Responses were recorded with sharp, intracellular electrodes as they were previously in the intact DRG. Additional patch-clamp studies were conducted to examine the ionic mechanisms underlying the excitatory effects of MCP-1. Preliminary results were previously published in abstract form (Sun et al. 2005).

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## METHODS

*Chronic compression of the DRG and labeling of neurons*

The animal experiments were approved by the Animal Care and Use Committee of Yale University School of Medicine and were in accordance with the guidelines provided by the National Institutes of Health. The CCD model was performed in 30 female Sprague-Dawley rats (130–180 g) using a previously described procedure (Hu and Xing 1998; Song et al. 1999). Briefly, under anesthesia with pentobarbital sodium (40 mg/kg, administered intraperitoneally [ip]), the ipsilateral, right transverse process and intervertebral foramina of L4 and L5 were exposed and a stainless steel L-shaped rod (0.63 mm diameter, 4 mm length) was inserted into each foramen, one at L4 and the other at the L5 ganglion. Cutaneous afferent neurons were retrogradely labeled by injecting Fluorogold (FG) solution in saline (1%, 0.05 ml; Molecular Probes, Eugene, OR) subcutaneously into the right lateral plantar region (Yao et al. 2003). An antibiotic, Baytril (enrofloxacin, 2.5 mg/kg administered intramuscularly, Bayer HealthCare LLC, Shawnee Mission, KS), was administered immediately after surgery. Five unoperated rats were used as controls for sharp-electrode intracellular recording experiments.

*Neuronal dissociation and culture*

Five to 7 days after CCD surgery, the rats were deeply anesthetized with pentobarbital (40 mg/kg, ip) and the L4 and L5 lumbar DRGs were exposed. Because all the rods were in the correct position (Song et al. 1999), all 60 DRGs from CCD animals were accepted for the experiments. DRG neurons were dissociated and cultured using a previously reported modified method (Ma et al. 2005; Yao et al. 2003). Briefly, isolated DRG were dissected free of adherent connective tissue and placed in complete saline solution (CSS) for cleaning and mincing. The CSS contained (in mM): 137 NaCl, 5.3 KCl, 1 MgCl<sub>2</sub>, 3 CaCl<sub>2</sub>, 25 Sorbitol, and 10 Hepes, adjusted to pH 7.2 with NaOH. The DRGs were then digested for 25 min with collagenase A (1 mg/ml; Boehringer Mannheim, Mannheim, Germany) and, for another 25 min, with collagenase D (1 mg/ml; Boehringer Mannheim) and papain (30 units/ml, Worthington Biochemical, Lakewood, NJ) in CSS containing 0.5 mM EDTA and 0.2 mg/ml cysteine at 37°C. The cells were dissociated by trituration in culture medium containing 1 mg/ml bovine serum albumin and 1 mg/ml trypsin inhibitor (Boehringer Mannheim) and plated on glass coverslips coated with 0.1 mg/ml polyornithine and 1 mg/ml laminin (Boehringer Mannheim). The culture medium contained equal amounts of Dulbecco's modified Eagle medium and F12 (Gibco, Grand Island, NY) with 10% FCS (HyClone Laboratories, Logan, UT) and 1% penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Life Technologies, Rockville, MD). The cells were incubated at 37°C (5% CO<sub>2</sub> balanced air) for 1 h after which culture medium without the trypsin inhibitor was added. All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Coverslips with adherent DRG neurons were placed in a recording chamber that was mounted on the stage of an upright microscope (BX50-WI, Olympus Optical, Tokyo) and viewed with a  $\times 40$  water immersion objective under differential interference contrast.

*Criteria for selection of neurons*

Twelve to 18 h after dissociation, FG-labeled DRG neurons of small diameter (22–30  $\mu\text{m}$ ) were selected for electrophysiological recordings. In those neurons for which intracellular or current-clamp studies were performed, and action potentials recorded, only those neurons that responded to capsaicin applied at the end of the experiment and/or had an inflection on the falling phase of the action potential and a resting membrane potential (RMP) equal to or more negative than  $-50$  mV were included in the study. From a total of 184 cells that were tested for a response to 1  $\mu\text{M}$  capsaicin, 48 showed no

responses. Because inward currents were blocked in recordings of voltage-dependent outward currents, a positive FG label and small diameter were used as acceptance criteria for these recordings.

*Intracellular recording*

The intracellular recording electrode was fabricated from borosilicate glass (World Precision Instruments, Sarasota, FL), pulled on a Flaming/Brown micropipette puller (P-97, Sutter Instrument, Novato, CA), and filled with 1 M KCl. Satisfactory recordings were obtained with electrode impedances of 60–80 M $\Omega$ . Electrophysiological data were collected under current clamp (MultiClamp 700A, Axon Instruments, Union City, CA), stored digitally by a Digidata 1320A interface, and analyzed off-line with pClamp 9.0 software (Axon Instruments). Signals were filtered at 2 kHz and digitized at 20 kHz. The bridge balance and capacity compensation were adjusted to minimize the transient voltage change at the start of current injection.

The experimental conditions under which intracellular recordings were obtained were identical to those of a prior study of the intact DRG (White et al. 2005). The recording chamber was perfused continuously at a rate of 2–3 ml/min with a bath solution of artificial cerebrospinal fluid (ACSF) preheated to  $36 \pm 0.5^\circ\text{C}$  by means of an in-line heater with a controller (TC-344A, Warner Instruments, Hamden, CT). The ACSF contained (in mM) 130 NaCl, 24 NaHCO<sub>3</sub>, 3.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, 1.2 CaCl<sub>2</sub>, and 10 dextrose was bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The pH of the solution was 7.4 and the osmolarity, 290–310 mOsm.

*Whole cell recording*

Based on previous observations that in the intact DRG from previously unoperated (control) rats, few neurons exhibited the receptor for MCP-1 (CCR2) or responded to MCP-1 application, whole cell recordings were performed only in neurons obtained from CCD ganglia. Whole cell recordings were obtained with conventional procedures at room temperature under either voltage-clamp mode, for recordings of isolated currents, or current-clamp mode for recordings of action potentials and membrane potentials (MultiClamp 700A; Axon Instruments) using pClamp 9 software (Axon Instruments). Electrodes were fabricated from borosilicate glass (World Precision Instruments) and pulled on a Flaming/Brown micropipette puller (Sutter P-97; Sutter Instrument). The pipette solution contained (in mM): 120 K-methane sulfonate, 10 KCl, 5 NaCl, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 11 EGTA, 10 HEPES, 2 Mg-ATP, and 1 Li-GTP. The pH 7.2 of the solution was adjusted with Tris-base and the osmolarity (310 mOsm) adjusted with sucrose. The impedance of a typical patch pipette was 2–4 M $\Omega$ . The electrophysiological recordings were filtered at 3 kHz and digitized at 20 kHz. The series resistance was compensated at 40–80%. The recording chamber was perfused continuously at a rate of 2–3 ml/min with a bath solution containing (in mM) 145 NaCl, 3 KCl, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 Hepes, and 10 glucose and adjusted to a pH of 7.4 and osmolarity of 300–310 mOsm. For K<sup>+</sup> current recordings, the bath solution contained (in mM): 145 *N*-methyl-D-glucamine, 3 KCl, 2.5 CdCl<sub>2</sub>, 0.6 MgCl<sub>2</sub>, 10 Hepes, and 10 glucose, adjusted to pH 7.4 using HCl.

*Chemical application*

All chemicals were from Sigma-Aldrich unless otherwise stated. Monocyte chemoattractant protein-1 (MCP-1) was purchased from R&D Systems (Minneapolis, MN). Lyophilized proteins were aliquoted in 0.1% bovine serum albumin in phosphate-buffered saline (BSA) and stored at  $-20^\circ\text{C}$  for  $<3$  wk before use. A concentration of 100 nM of MCP-1 in BSA was prepared just before each day's experiments. The BSA vehicle and MCP-1 were each delivered directly to the soma of each recorded neuron through a pipette with a 100- $\mu\text{m}$ -diameter tip and a fast-switch, pressure-controlled drug ap-

plication system (Automate, Fullerton, CA). The tip of the pipette was located about 100  $\mu\text{m}$  away from the neuron studied. Only one neuron per coverslip was used for study.

### Statistical analyses

The error bars for the means presented in each figure are means  $\pm$  SE. Comparisons of proportions were made using the chi-square test or Fisher's Exact test. In the case of multiple comparisons, for instance, over a voltage range, data were initially analyzed using two-way repeated-measures ANOVA. If this revealed a significant difference, paired *t*-tests were applied for post hoc testing with a Bonferroni correction applied to the critical *t* value. Changes in the action potential parameters were compared between baseline and treatment conditions using a paired-*t*-test. Differences between groups in the excitability studies were determined using a *t*-test or a Mann-Whitney rank sum test (if the data failed a normality or equal variance test). Statistical significance was established at  $P < 0.05$ . Origin 6.0 (Microcal Software, Northampton, MA) was used for curve fitting. The voltage dependency of activation of  $\text{K}^+$  conductance was fitted with the Boltzmann function of the form:  $G/G_{\text{max}} = 1/(1 + \exp[V_{1/2} - V_m/k])$ , where  $G$  is the conductance,  $G_{\text{max}}$  is the fitted maximal conductance,  $V_{1/2}$  is the membrane potential for half activation,  $V_m$  is the command potential, and  $k$  is the slope factor. The concentration-response curve was constructed using a nonlinear-regression analysis.

## RESULTS

### MCP depolarized the resting membrane potential and altered action-potential properties

Intracellular recordings were obtained from 19 neurons from control DRGs (obtained from unoperated rats) and 20 neurons from CCD ganglia. Local, puff application of MCP-1 (100 nM, 1 min) but not vehicle (data not shown), depolarized ( $\geq 2$  mV) the majority of CCD neurons (15 of 20; 75%) but only a few of the control cells (2 of 19; 10.5%) (Fig. 1A). The mean magnitude of depolarization of the responsive CCD neurons was  $15.5 \pm 4.0$  mV (range: 4–33.8 mV). Four of 20 responsive neurons (20%) exhibited spontaneous action potentials; these neurons had a mean depolarization of  $24.5 \pm 7.4$  mV (Fig. 1B). After a washout of MCP-1, the membrane potential returned to, or approached, its basal value. The mean magnitude of depolarization to MCP-1 was significantly greater for the acutely dissociated CCD neurons (present study) than for neurons previously recorded from the intact DRG ( $15.5 \pm 4.0$  vs.  $4.0 \pm 1.4$  mV,  $P < 0.001$ , two-tailed *t*-test), although the proportions of neurons that depolarized were similar (15/20 vs. 6/10,  $P = 0.76$ , Fisher's Exact test, Fig. 1C). Similarly, the proportion of control neurons responsive to MCP-1 was not significantly different from that of the intact ganglion (White et al. 2005) (2/19 vs. 0/10,  $P = 1$ , Fisher's Exact test).

The greater depolarization induced by MCP-1 of dissociated neurons as opposed to neurons in the intact DRG may be attributable, in part, to the effect of the dissociation process, per se, in increasing the excitability of small diameter neurons (Ma et al. 2005). The dissociation and separation of cells may also afford greater access of MCP-1 to the recorded neuron.

To investigate further the effect of MCP-1 in facilitating the generation of action potentials, we measured the rheobase, accommodation, and the action potential threshold before and during exposure to MCP-1 (Fig. 2). A series of increasing current steps, each of 500-ms duration, were delivered. The rheobase (current threshold) was defined as the minimal cur-

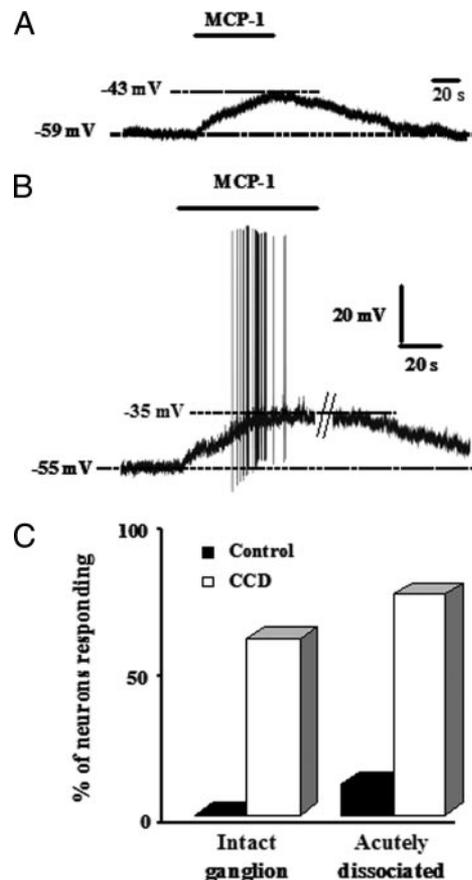


FIG. 1. Monocyte chemoattractant protein-1 (MCP-1) depolarized the resting membrane potential. Intracellular recordings were made of the responses of each neuron to a 1-min application of MCP-1 (100 nM). *A*: depolarization of a typical chronic compression of the dorsal root ganglion (CCD) neuron by MCP-1. *B*: action potentials elicited by MCP-1 in a typical neuron. MCP-1 evoked discharges in 4 of 20 neurons tested. *C*: comparison of the percentage of neurons responding to MCP-1 in the intact dorsal root ganglion (DRG) and in acutely dissociated DRG neurons. Data for the intact DRG were obtained previously (White et al. 2005). There were no significant differences between percentages obtained for neurons in the 2 groups under either control conditions (solid bars) or after CCD (open bars). Proportion of neurons depolarized was significantly greater for CCD than for control neurons regardless of whether they were acutely dissociated or in the intact DRG.

rent eliciting an action potential. MCP-1 significantly induced a decrease in the mean rheobase by 61.55% ( $P < 0.05$ , two-tailed paired *t*-test,  $n = 8$ ) and increased by 7.7-fold the number of action potentials evoked by a current injection at an amplitude of twice the rheobase ( $P < 0.05$ , one-tailed paired *t*-test,  $n = 6$ ). The action potential voltage threshold, defined as the first point on the upstroke of an action potential where the rising rate exceeded 50 mV/ms (Anderson et al. 1987), was significantly lower (more negative) during the application of MCP-1 ( $-32.5 \pm 1.5$  vs.  $-27.7 \pm 1.3$  mV,  $P < 0.001$ , two-tailed paired *t*-test,  $n = 9$ ). The changes in rheobase, the number of evoked action potentials, and action potential threshold did not recover for  $\geq 10$  min after washout of MCP-1. These results reveal excitatory effects of MCP-1 in addition to the depolarization of the resting membrane potential.

To examine the effects of MCP-1 on the shape of the action potential, a current injection of suprathreshold amplitude and 2-ms duration was delivered under current clamp before and

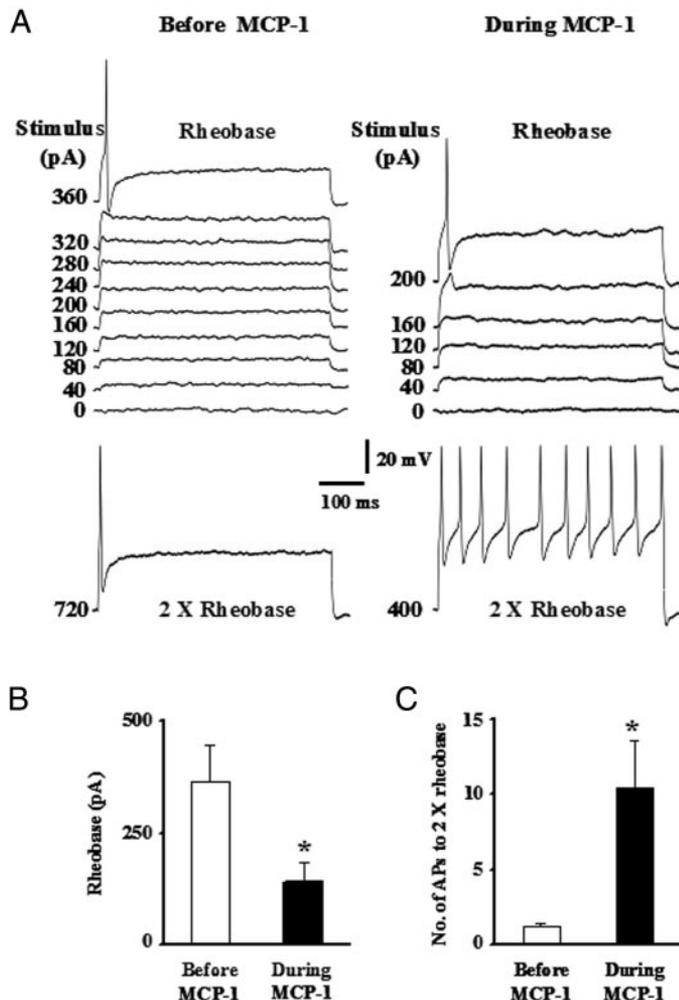


FIG. 2. MCP-1 reduced the rheobase and accommodation in response to current injection. Intracellular recordings were made of the responses of each neuron to a 1-min application of MCP-1 (100 nM). *A*: MCP-1 decreased the rheobase (threshold current required to evoke an action potential) in a typical neuron, while decreasing its accommodation, i.e., increasing the number of action potentials evoked by current equal to twice the rheobase. *B*: mean rheobase before and during the application MCP-1 ( $n = 8$ ). *C*: mean number of action potentials (APs) evoked by a current injection of 2  $\times$  rheobase before and during application of MCP-1 ( $n = 6$ ). (\* $P < 0.05$ ).

during the application of MCP-1. The effects are summarized in Table 1. MCP-1 significantly broadened the mean duration of the action potential at voltages equal to both 50 and 10% of the peak amplitude (Fig. 3). The action potential had not returned to its original duration 10 min after washout of MCP-1 (data not shown,  $n = 4$ ). Further analyses revealed that MCP-1 induced a significant increase in the mean fall time of the action potential from 90 to 10% of the peak action potential

TABLE 1. Effects of MCP-1 on the shape of the action potential

	APD <sub>50</sub> , ms	APD <sub>10</sub> , ms	AP RT, ms	AP FT, ms	AP Rate of Rise, mV/ms	AP Rate of Fall, mV/ms	AHP <sub>50</sub> Amplitude, mV	AHP <sub>50</sub> Duration, ms
Before MCP-1	2.31 $\pm$ 0.16	4.94 $\pm$ 0.23	2.43 $\pm$ 0.12	3.23 $\pm$ 0.29	34.32 $\pm$ 1.10	37.76 $\pm$ 3.73	21.63 $\pm$ 0.84	26.52 $\pm$ 3.23
During MCP-1	2.68 $\pm$ 0.20***	5.90 $\pm$ 0.47**	2.58 $\pm$ 0.12	4.35 $\pm$ 0.49*	33.16 $\pm$ 1.30	32.18 $\pm$ 3.43**	20.12 $\pm$ 1.19*	23.78 $\pm$ 2.68*

Values are means  $\pm$  SE;  $n = 12$  neurons. APD<sub>50</sub>, duration at 50% of the peak amplitude; APD<sub>10</sub>, duration at 10% of the peak amplitude; AP RT, the action potential rise time; AP FT, the action potential fall time; AHP<sub>50</sub> Amplitude, afterhyperpolarization amplitude to 50% recovery; AHP<sub>50</sub> Duration, afterhyperpolarization duration to 50% recovery. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  indicate significant differences between "before" and "during" MCP-1 application (two-tailed  $t$ -tests).

and a significant decrease in the rate of fall (Table 1). In contrast, MCP-1 had no significant effect on the time or rate of the rising phase of the action potential. Thus, MCP-1 increased the duration of the action potential by prolonging its falling phase.

The magnitude of afterhyperpolarization (AHP) was measured from the resting membrane potential (RMP) to the most hyperpolarized value of the AHP. The duration of the AHP was measured at points representing 50% recovery of the AHP back to RMP (AHP<sub>50</sub>). In comparison with values obtained before application, MCP-1 significantly decreased both the mean magnitude and the mean duration of the AHP (Table 1).

#### MCP induced an inward current

To define the ionic currents underlying MCP-1-evoked depolarization and hyperexcitability in nociceptive CCD neurons, whole cell voltage-clamp recordings were performed before and during MCP-1 application. Puff application of MCP-1 induced an inward current in 27 of 50 neurons (held at  $-60$  mV), a proportion not statistically different from the proportion of neurons depolarized by MCP-1 during recordings with intracellular electrodes (chi-square test,  $P = 0.177$ ). Typically, the inward current was evoked immediately after MCP-1 application, reached its peak in about 1 min, and recovered after washout. The magnitude of the MCP-1-induced inward current was  $67 \pm 57$  pA ( $n = 27$ ) and was always accompanied by a depolarization under current clamp in the same neuron (Fig. 4, *A* and *B*). These MCP-1 responses remained in the presence of tetrodotoxin (TTX, 1  $\mu$ M) (data not shown,  $n = 5$ ).

The MCP-1-induced inward current was concentration dependent over a range of 3 to 300 nM as determined by applying different concentrations of MCP-1 to different groups of neurons (Fig. 4*C*). The effective concentration eliciting half of the maximal response (EC<sub>50</sub>) was 45 nM.

#### MCP activated a current with properties consistent with a nonselective cationic conductance

It is well established that some peptides excite primary sensory neurons by activating a nonselective cationic conductance (NSCC) (Inoue et al. 1995; Li et al. 1998). To determine whether MCP-1 activates a NSCC, steady-state current-voltage relationships were determined using a voltage-ramp protocol (Fig. 5*A*, inset). MCP-1-induced current was obtained for each neuron by subtracting the response to a voltage ramp (120 mV/s) in the absence of MCP-1 from the response to the same ramp evoked during the application of MCP-1 (Fig. 5*B*). The current-voltage relationship for the mean MCP-1-activated currents is linear throughout the voltage range tested, indicat-

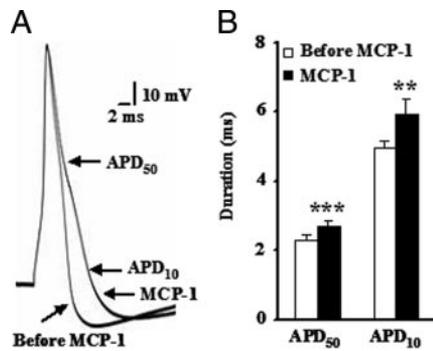


FIG. 3. MCP-1 increased the duration of the action potential. Whole cell current-clamp recordings were made of the responses of CCD neurons to short current injections of 2 ms at rheobase before and during 1-min application of MCP-1 (100 nM). *A*: action potentials evoked in a typical CCD neuron before and during the application of MCP-1 (100 nM). Duration of the action potential was increased at amplitudes that were both 50 and 10% of the peak (APD<sub>50</sub> and APD<sub>10</sub>, respectively). *B*: mean durations of the action potential taken at APD<sub>50</sub> and APD<sub>10</sub> before and during application of MCP-1 (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 12$ ).

ing a lack of voltage dependency (Fig. 5, *B* and *C*). This voltage-independent conductance is characteristic of a NSCC (Bourque 1989; Desson et al. 2003; Hiruma and Bourque 1995; Kirkpatrick and Bourque 1995; Yang and Ferguson 2002, 2003; Yang et al. 2004). The MCP-1-sensitive current reverses at  $-27 \pm 3$  mV ( $n = 11$ ). Based on the mean input resistance for our CCD neurons of 309 M $\Omega$  ( $n = 30$ ), at a holding potential of  $-60$  mV, the calculated MCP-1-induced inward current would be expected to evoke a nearly 18-mV depolarization—a value very close to the observed mean depolarization of  $16.5 \pm 3.5$  mV evoked by 100 nM MCP-1 under whole cell current-clamp recordings ( $n = 12$ ). Furthermore, in three neurons held at  $-30$  mV, a voltage close to the reversal potential of this NSCC, no noticeable inward currents were evoked by MCP-1 (100 nM). In contrast, larger inward currents were evoked by the repeated application of MCP-1 (100 nM) to neurons held at  $-90$  mV compared with values from the same cells held at  $-60$  mV ( $n = 4$ ). Because the reversal potential for the MCP-1-sensitive current was positive to the calculated Nernst potential for chloride ( $E_{Cl} = -50$  mV), it is unlikely that the current is mediated by an anion conductance (Baba et al. 2001). These data are consistent with MCP-1 activating a NSCC in CCD neurons.

#### MCP inhibited the voltage-dependent, noninactivating $K^+$ conductance ( $I_K$ )

The MCP-1-induced reduction in rheobase, together with an increase in spike frequency and a broadening of action potentials, suggests that MCP-1 might reduce a  $K^+$  conductance. We therefore performed whole cell voltage-clamp recordings to test this hypothesis. In view of the complexity and variability of  $K^+$  currents in DRG neurons (Akins and McCleskey 1993; Gold et al. 1996a), we performed a simplified analysis of the effects of MCP-1 on the two kinetically distinct voltage-dependent  $K^+$  currents identified previously in DRG neurons: a fast inactivating  $K^+$  current ( $I_A$ ) and a voltage-dependent, noninactivating  $K^+$  current ( $I_K$ ) (Akins and McCleskey 1993; Everill et al. 1998; Gold et al. 1996a; McFarlane and Cooper 1991). We initially examined the effects of MCP-1 on peak whole cell  $K^+$  currents evoked by 10-mV depolarizing voltage

steps, each 400 ms, from  $-120$  to  $+60$  mV from a holding potential of  $-120$  mV before and during application of MCP-1. Exposure to MCP-1 (100 nM) substantially reduced the net whole cell  $K^+$  currents ( $n = 19$ ;  $df = 1, 18$ ;  $F = 14.96$ ;  $P < 0.001$ , two-way repeated-measures ANOVA) as illustrated for one neuron in Fig. 6*A* and the mean net whole cell  $K^+$  currents obtained for all neurons in Fig. 6*B*. The MCP-1-induced decrease in net whole cell  $K^+$  currents, in relation to base values obtained before chemical application, began with the  $-10$ -mV step and progressively increased up to the  $+60$ -mV step (two-tailed paired  $t$ -test,  $P < 0.05$ , using Bonferroni correction, Fig. 6*B*). Normalization of the peak current evoked from each command potential to the maximal current amplitude made it possible to obtain an activation curve of net whole cell  $K^+$  current (Fig. 6*C*). MCP-1 treatment did not modify the current-voltage relationship of net whole cell  $K^+$  current. There was no significant difference in the mean half-

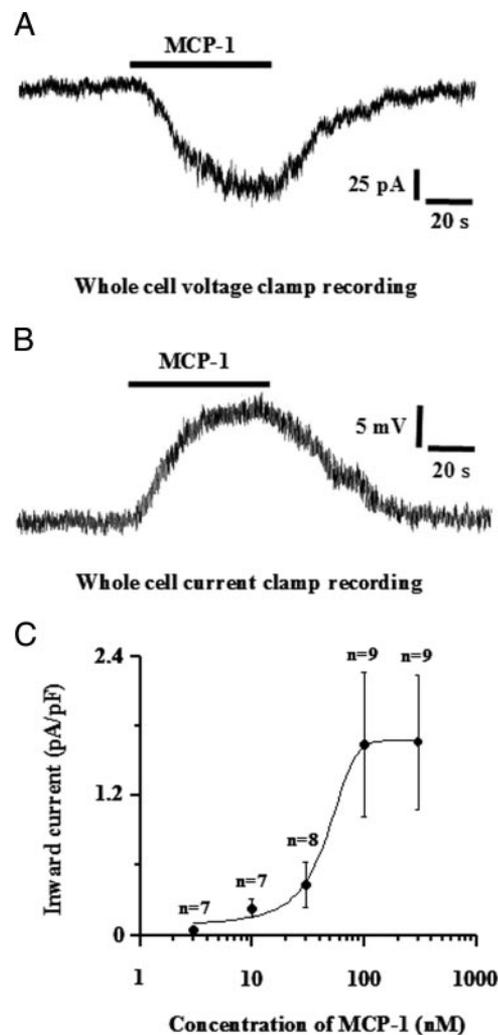


FIG. 4. MCP-1 evoked an inward current. *A*: ionic currents activated by MCP-1 recorded in a typical neuron under voltage clamp (holding at  $-60$  mV). *B*: depolarization by MCP-1 recorded in the same neuron under current-clamp mode. *C*: mean peak amplitude current increased with the concentration of MCP-1 (each mean  $\pm 1$  SE was obtained from the stated sample size,  $n$ ). Each peak current value was taken at the end of the (1-min) period of chemical application. Software (Origin 6.0) was used to fit a curve to the means to obtain a concentration-response function. Effective concentration eliciting half of the maximal response ( $EC_{50}$ ) was 45 nM.

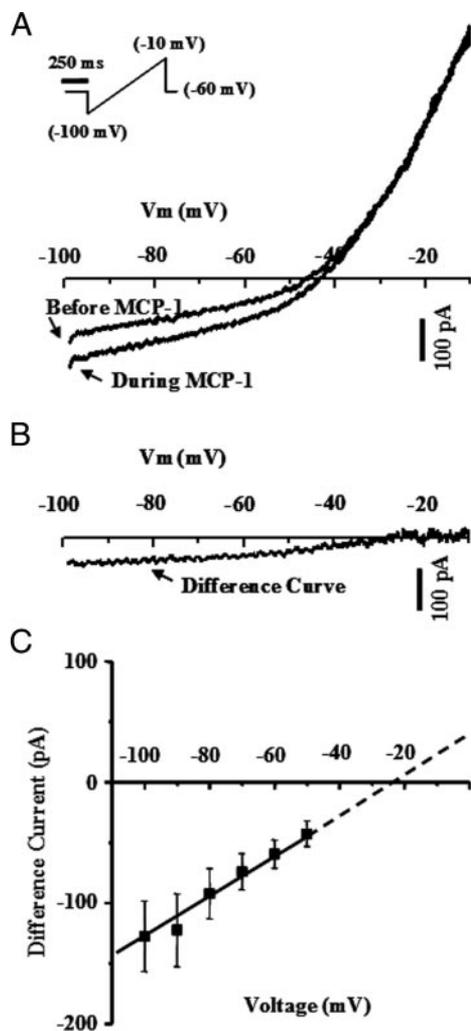


FIG. 5. MCP-1-induced inward current exhibited characteristics of a non-selective cationic conductance (NSCC). *A*: whole cell currents evoked from a rapidly depolarizing voltage ramps (120 mV/s) (*inset*) before and during exposure to MCP-1 (100 nM). *B*: a difference current was obtained by subtracting the amplitudes of the current evoked before MCP-1 from the current elicited during the application of MCP-1. Difference current represented the inward current evoked by MCP-1. *C*: mean current evoked by MCP-1 in responsive neurons. Dashed line substitutes for currents corresponding to voltages positive to  $-50$  mV that triggered a different (outward) current.

activated conductance values obtained before and during MCP-1 application ( $-3 \pm 1$  and  $-4 \pm 1$  mV,  $P > 0.05$ , one-tailed paired-*t*-test,  $n = 19$ ).

The effects of MCP-1 on the voltage-dependent, noninactivating  $K^+$  current were tested using a voltage protocol that inactivated  $I_A$  and thus permitted a direct assessment of  $I_K$ . Voltage steps, each of 400-ms duration and 10-mV increment, of  $-120$  to  $+60$  mV were delivered from a prepulse ( $-30$  mV) before and during the application of MCP-1 (100 nM) and  $I_K$  measured at the end of each step. MCP-1 significantly decreased  $I_K$  ( $n = 19$ ;  $df = 1,18$ ;  $F = 30.50$ ;  $P < 0.001$ , two-way repeated-measures ANOVA) as illustrated for the responses of a typical neuron in Fig. 7*A* and for the mean  $I_K$  for all neurons in Fig. 7*B*. The decrease in mean  $I_K$  occurred in response to depolarizing pulses more positive than  $-10$  mV (two-tailed paired *t*-test,  $P < 0.05$ , using Bonferroni correction, Fig. 7*B*). There was no significant difference in the half-activated conductance values for the averaged activation curve of  $I_K$  ob-

tained before and during MCP-1 ( $-5 \pm 1$  and  $-7 \pm 1$  mV, respectively,  $n = 19$ ,  $P > 0.05$ , one-tailed paired *t*-test), indicating that MCP-1 had no effects on the activation curve of  $I_K$  (Fig. 7*C*). No recovery was seen from the MCP-1-induced decreases in net whole cell  $K^+$  current or voltage-dependent, noninactivating  $K^+$  currents in the time course of our recordings. This effect was unlikely to be the result of current rundown because when the same voltage protocol was delivered twice to CCD neurons, in the absence of MCP-1, there was no reduction in either  $I_K$  or  $I_A$  (repeated-measures ANOVAs,  $n = 15$ ;  $df = 1,14$ ;  $F < 0.4$ , and  $P > 0.9$ ).

MCP had no effect on the fast inactivating  $K^+$  current ( $I_A$ )

$I_A$  was isolated for each neuron by subtracting  $I_K$  from the net whole cell  $K^+$  current. The amplitude of  $I_A$  was measured at the peak of the subtracted current (Fig. 8*A*). MCP-1 had no significant effect on the amplitude or activation parameters of  $I_A$  ( $n = 19$ ;  $df = 1,18$ ;  $F = 0.31$ ;  $P = 0.5805$ , two-way repeated-measures ANOVA; Fig. 8, *B* and *C*). Values of half-activated conductance for the fast inactivating  $K^+$  currents were  $7 \pm 4$  and  $3 \pm 2$  mV in the absence and presence of MCP-1, respectively ( $P > 0.05$ , one-tailed paired *t*-test,  $n = 19$ ).

## DISCUSSION

The excitatory effects of MCP-1 on acutely dissociated, CCD-treated, small-diameter, nociceptive neurons are similar

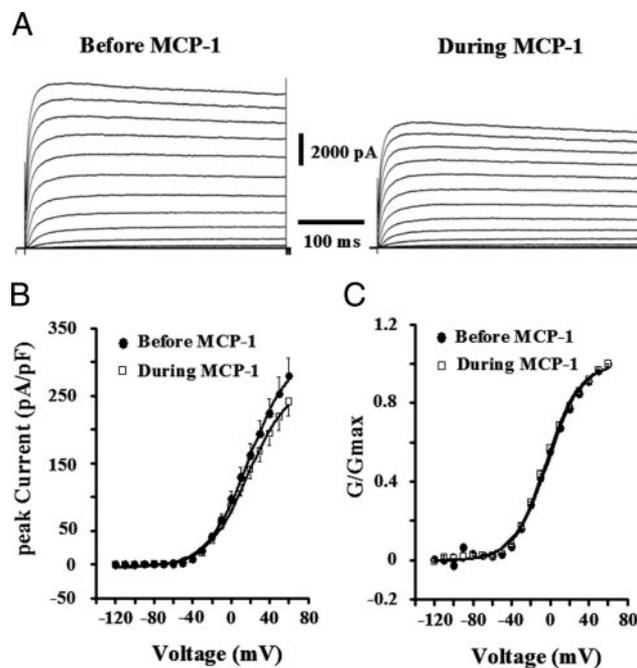


FIG. 6. MCP-1 decreased the net whole cell  $K^+$  current. Net whole cell  $K^+$  current was recorded from CCD neurons in response to voltage steps (400-ms duration, 10-mV increments) between  $-120$  and  $60$  mV from a holding potential of  $-120$  mV. *A*: net whole cell  $K^+$  currents (leak subtracted) recorded from a typical neuron. Currents were recorded before (*left*) and during (*right*) a 1-min application of MCP-1 (100 nM). *B*: mean current evoked by each voltage before (solid circles) and during (open squares) the application of MCP-1. A significant inhibition of peak current by MCP-1 occurred in response to all voltages more positive to  $-10$  mV. *C*: mean activation curves obtained before (solid circles) and during 100 nM MCP-1 (open square). MCP-1 did not shift the activation curve of the net whole cell  $K^+$  current.

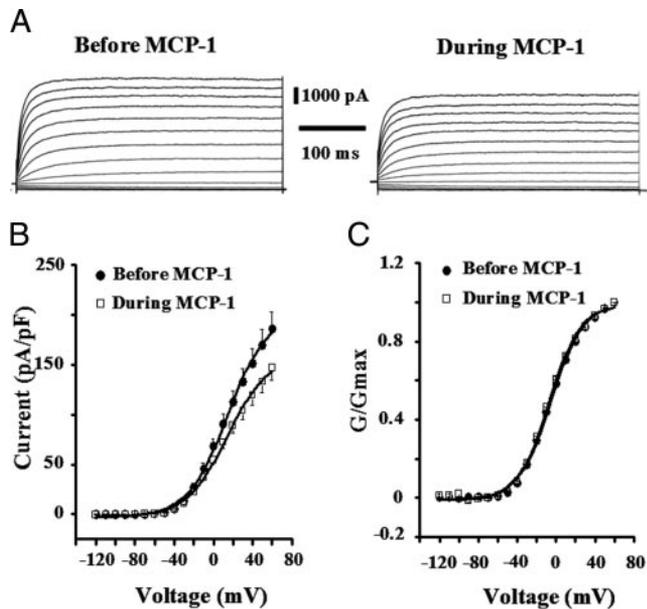


FIG. 7. MCP-1 decreased the sustained  $K^+$  current. Sustained current, recorded under voltage clamp, was evoked by applying voltage steps (each 400 ms) in increments of 10-mV steps from  $-120$  to  $60$  mV from a holding potential of  $-30$  mV. Responses were obtained from the same neurons for which the net whole cell  $K^+$  current was evoked and plotted in the same format as in Fig. 6. *A*: sustained  $K^+$  current for the neuron whose responses are shown in Fig. 6*A*. *B*: mean sustained current as a function of the voltage of each step delivered before (solid circles) and during (open squares) MCP-1. Value of the current for each neuron was obtained at the end of the step. Inhibitory effect of MCP-1 was significant positive to the  $-10$ -mV step. *C*: mean steady-state activation obtained before (solid circles) and during 100 nM MCP-1 (open squares). MCP-1 did not shift the activation curve of the sustained  $K^+$  current.

to those previously observed in small-diameter neurons in the intact, CCD-treated DRG (White et al. 2005). In neurons of dissociated ganglia, MCP-1 depolarized the membrane potential and increased excitability as evidenced by evoked discharges or by a reduction in the current threshold for the generation of action potentials. Furthermore, MCP-1 evoked an inward current that mediated its depolarizing effects on these neurons. Because the excitatory action of MCP-1 was observed in the absence of neighboring cells and/or substances they might release (which are likely to be washed away by the bath perfusion), we conclude that MCP-1 has a direct excitatory effect on CCD sensory neurons. This does not preclude the possibility that, in the intact ganglion, MCP-1 might also have indirect effects by the release of substances from neighboring cells.

MCP-1 preferentially binds to CCR2 (Rossi et al. 2000). The responsiveness of nociceptive DRG neurons to CCR2 was previously confirmed by  $Ca^{2+}$  flux in the presence of MCP-1 (Oh et al. 2001). Recently, we found that mRNA for CCR2, rarely present in control DRG neurons, was upregulated in the cell bodies of subpopulations of nociceptive and nonnociceptive CCD neurons in the intact DRG (White et al. 2005). Although the lack of specific CCR2 antagonists precluded the identification of the CCR2 receptor mediating these effects, the clear reversibility and concentration dependency of these effects shown in this study, together with the known presence of CCR2 mRNA in CCD neurons, argue strongly that they are receptor mediated.

Chemokines have been implicated in different neuronal functions in both normal and pathological conditions. Thus, the acute regulation of ion channels by chemokines has been a subject of considerable interest (Bordey et al. 2003; Puma et al. 2001; Rappert et al. 2002). CCR4/MDC (macrophage-derived chemokines), for example, has been shown to elicit a rapid, inhibitory effect on calcium channels in subpopulations of dorsal root ganglion neurons (Oh et al. 2002). Chemokine receptors are G-protein-coupled, seven-transmembrane receptors. Activation of various G-protein-coupled receptors modulates different ion channels by various signaling pathways. MCP-1 binds to and signals through a seven-transmembrane-spanning  $G\alpha i$ -protein-coupled receptor, CCR2.

Although G-protein-coupled receptor activation of ion channels has been extensively investigated (Dascal 2001; Wickman et al. 1995), only a few ionic conductances modulated by CCR2 receptor activation have been described (Goselin et al. 2005). In this study, we demonstrate that the CCR2-mediated depolarization is likely a result of the activation of a nonselective cationic conductance (NSCC) by MCP-1 in a concentration-dependent manner. A NSCC is a cationic-selective, membrane channel that is permeable to  $Na^+$ ,  $K^+$ , or  $Ca^{2+}$ . One of the striking biophysical features of NSCCs is their voltage independence. The evidence for activation of a nonspecific cation conductance by MCP-1 was based on the following three observations. First, the MCP-1-induced inward current reversed at about  $-27$  mV. Therefore, it is unlikely that the MCP-1 response is mediated solely by  $K^+$ ,  $Cl^-$ , or  $Na^+$  channels ( $E_K = -95$  mV,  $E_{Cl} = -50$  mV,  $E_{Na} = 85$  mV). Inclusion of TTX in the external solution did not affect the MCP-1-induced current, thereby excluding the involvement of fast  $Na^+$  channels in the MCP-1-induced current in the recorded CCD neurons. Second, based on the linear current-voltage relationship of MCP-1-induced currents, val-

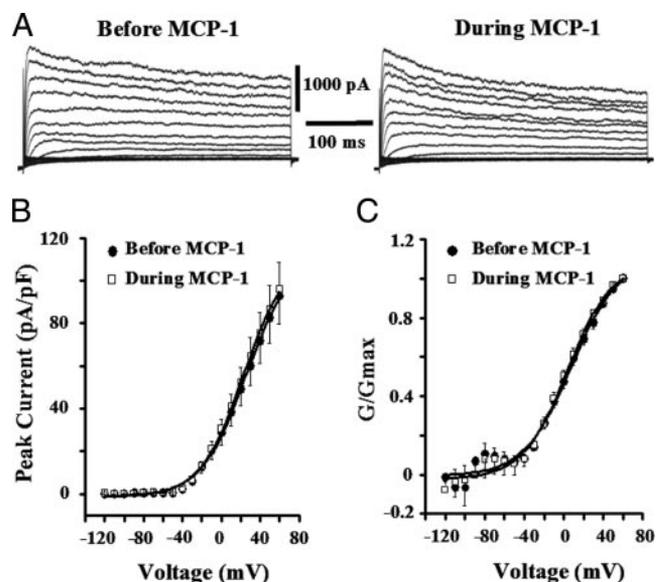


FIG. 8. MCP-1 did not alter the fast inactivating  $K^+$  current. Traces were obtained by off-line subtraction of the sustained (Fig. 7) from the total (Fig. 6). Same format as in Figs. 6 and 7. *A*: fast inactivating  $K^+$  current for a typical neuron. *B*: mean peak fast inactivating  $K^+$  current evoked by each test voltage before and during MCP-1. Current was not significantly altered by MCP-1. *C*: mean activation curves for the fast inactivating  $K^+$  current obtained before (solid circles) and during 100 nM MCP-1 (open squares). Activation curve was not significantly altered by MCP-1.

ues of the predicted depolarization of MCP-1 are in accordance with the potential changes in response to MCP-1 application recorded in current-clamp experiments. Third, MCP-1-induced currents increased in amplitude when the holding potential was lowered to  $-90$  mV from  $-60$  mV but were not detected when the cells were held at  $-30$  mV, a potential close to the reversal potential of this NSCC ( $-27$  mV). Collectively, these data suggest that the MCP-1-induced depolarization is mediated by a NSCC.

A membrane depolarization mediated through the activation of NSCCs in DRG neurons is not unique to MCP-1. Heat, capsaicin, and other stimuli depolarize a subset of nociceptive DRG neurons by activating a NSCC by the transient receptor potential vanilloid 1 (TRPV1) (Oh et al. 1996). Inflammatory mediators such as substance P (SP), prostanoid PGE<sub>2</sub>, and bradykinin (BK) were shown to be involved in activating NSCCs. For example, SP opens NSCCs in DRG neurons through the activation of NK receptors (Inoue et al. 1995; Li et al. 1998). PGE<sub>2</sub>-induced depolarization, mediated through the activation of a NSCC, underlies a direct postsynaptic excitatory action on deep dorsal horn neurons (Baba et al. 2001). BK activation of lipoxygenases produces lipid metabolites that stimulate TRPV1 receptors leading to the opening of NSCCs (Shin et al. 2002). Although the cellular mechanisms underlying the induction of hyperalgesia by CXC chemokines IL-1 $\beta$  (Oprea et al. 2000) remain unclear, a recent study has shown that IL-1 $\beta$  modulates neuronal excitability in the subformical organ through the activation of a nonselective cation channel (Desson et al. 2003). Similar mechanisms for IL-1 $\beta$  might also exist in nociceptor activation.

Thus NSCCs in nociceptive neurons are implicated in nociception. The present evidence for a CCR2-mediated activation of NSCCs in CCD-injured neurons further illustrates the possible mechanisms underlying the excitation of neurons and pain behavior in CCD rats. The activation of G-protein-coupled receptors can regulate NSCCs through different intracellular signal pathways in various cells (Poyer et al. 1996; Yoshifumi et al. 2004). However, the intracellular activation mechanisms of NSCCs by MCP-1 in DRG neurons have yet to be described.

In addition to, and independent of, its depolarizing effects, MCP-1 also increased neuronal excitability by lowering current threshold and increasing the number of depolarization-induced action potentials evoked after repolarizing to the original membrane potential. MCP-1 also shifted the voltage threshold to more negative values and reduced the AHP. These properties contribute to the MCP-1-induced hyperexcitability in CCD neurons. An enhancement of neuronal excitability in the absence of membrane depolarization was previously described in dissociated DRG neurons during exposure to PGE<sub>2</sub>, which suppresses the sustained K<sup>+</sup> current (Nicol et al. 1997). Similarly, other proinflammatory mediators such as serotonin enhance the excitability of sensory neurons through the suppression of K<sup>+</sup> conductances (Klein et al. 1982; Siegelbaum et al. 1982). The inhibition of K<sup>+</sup> conductances is thought to mediate, at least in part, the hyperexcitability of axotomized DRG neurons (Abdulla et al. 2001; Everill et al. 1999). The candidate K<sup>+</sup> currents modulating excitability in small-sized, nociceptive DRG neurons are the sustained delayed rectifier type and the transient A-type K<sup>+</sup> currents ( $I_k$  and  $I_A$ , respec-

tively) (Gold et al. 1996a; Yang et al. 2004; Yoshimura et al. 1999).

The present results demonstrate that MCP-1 decreases  $I_k$  but not  $I_A$  current. In contrast to the MCP-1-induced inward currents that were clearly reversible, the decrease in  $I_k$  currents in CCD neurons remained after washout of MCP-1, indicating the activation of separate signal transduction pathways. Because neither the enhanced excitability nor the reduction in  $I_k$  currents was reversible, the enhanced excitability could be caused by the reduction in  $I_k$  currents. However, MCP-1 did not significantly alter  $I_k$  below a threshold of  $-10$  mV, which is above the voltage threshold for an AP, suggesting that the decrease in the action potential threshold by MCP-1 is not attributable to the MCP-1 mediated inhibition of  $I_k$ . Both voltage-gated sodium and K<sup>+</sup> channels play a fundamental role in controlling neuronal excitability (Hille 2001). The lowering of voltage threshold by MCP-1 might be mediated by changes in sodium channels. Small-diameter nociceptive sensory neurons express both TTX-S and TTX-R Na<sup>+</sup> channels (Elliott et al. 1993; Yoshimura et al. 1996) but the TTX-R Na<sup>+</sup> channels, especially Na<sub>v</sub>1.8, contribute substantially to action potential electrogenesis in nociceptive neurons (Blair and Bean 2002). A significant increase in TTX-R  $I_{Na}$  contributes in part to the hyperexcitability in sensory neurons innervating the inflamed ileum (Stewart et al. 2003). Inflammatory mediators such as PGE<sub>2</sub> and 5HT increased the excitability of nociceptive sensory neurons by modulating TTX-R  $I_{Na}$  (Cardenas et al. 2001; Gold et al. 1998). Moreover, mice deficient in Na<sub>v</sub>1.8 exhibit decreased neuronal excitability (Renganathan et al. 2001) and decreased visceral pain (Laird et al. 2002). Thus an enhancement of TTX-R Na<sup>+</sup> current is a possible or likely explanation for the enhanced excitability after MCP-1 but remains to be investigated.

Although the MCP-1-mediated inhibition of K<sup>+</sup> conductances may not mediate depolarization or the lowered voltage threshold for action potential generation, the reduced  $I_k$  might be responsible in part for the reduced AHP and prolongation of the AP. Previous studies showed that TEA, a blocker of some voltage-activated K<sup>+</sup> currents, reduced the amplitude of AHP in DRG neurons (Safronov et al. 1996). In various neurons, the magnitude and duration of the AHPs play important roles in setting neuronal firing rate. Several lines of evidence have shown that reduction of AHPs induces enhanced excitability of DRG neurons (Djoughri et al. 1998; Gold et al. 1996b). A reduction in AHP could explain our observation that more APs are generated for the same depolarizing current ( $2 \times$  rheobase) in the MCP-1-treated small nociceptive neurons. In addition, a decrease in (outward)  $I_k$  is expected to hinder repolarization of the AP, likely accounting for the broadening of the action potential observed during MCP-1 application.

Our data from the current study indicate that  $I_A$  currents contribute a relatively small portion to the net whole cell K<sup>+</sup> current in these nociceptive DRG neurons. This is consistent with previous data from our laboratory showing that CCD predominantly reduces  $I_A$  currents in DRG neurons (Tan et al. 2006), the mechanisms underlying which need further investigation. The present study provides evidence that  $I_k$  conductances in small CCD neurons are more sensitive than  $I_A$  to MCP-1 application. Combined with our previous data showing the upregulation of MCP-1 and its receptor CCR2 in nociceptive DRG neurons after CCD, the MCP-1-mediated suppres-

sion of  $I_k$  may contribute, at least in part, to the pain behavior and in vivo, neuronal hyperexcitability after CCD (Hu and Xing 1998; Song et al. 1999).

In addition to  $K^+$  currents, calcium and sodium currents have been implicated in determining the duration of the action potential. The block of calcium channels by the application of inorganic divalent ions such as  $Cd^{2+}$  and  $Co^{2+}$  reduced or even completely abolished the shoulder of action potential in small DRG neurons (Abdulla and Smith 1997; Villiere and McLachlan 1996; Yoshida et al. 1978). The MCP-1-induced slower fall time and longer duration of the action potential suggests an enhancement of calcium current. In addition, recent evidence showing the slow inactivation of TTX-R sodium current may contribute to the longer duration of action potentials of small, nociceptive DRG neurons (Blair and Bean 2002, 2003; Renganathan et al. 2001). Thus MCP-1 may affect TTX-R sodium current as well as calcium current.

Little is known about the CCR2-activated intracellular signal transduction mechanisms in nociceptive neurons during chronic pain conditions, but the activation of MAPK by MCP-1 has been reported in different cell types (Jimenez-Sainz et al. 2003; Ogilvie et al. 2003; Sodhi et al. 2002). Recent papers provide evidence that MAP kinases play a critical role in neuropathic pain. p38 MAPK activation in DRG neurons has been described in different neuropathic pain models (Jin et al. 2003; Kim et al. 2002; Obata et al. 2004; Schafers et al. 2003). The upregulation of pERK was observed in CCD neurons (preliminary unpublished data in our laboratory). Furthermore, activation of MAPK has been described as inhibiting  $K^+$  conductances in nociceptive neurons (Hu et al. 2003). Therefore MCP-1/CCR2 signaling might modulate ion channels through the activation of MAPK in DRG neurons, although further study is needed.

In conclusion, MCP-1 excites CCD-treated neurons through multiple pathways: 1) activation of a nonvoltage-dependent depolarizing current with characteristics consistent with a NSCC; 2) a reduction in sustained, voltage-dependent  $K^+$  currents; and 3) changes in action-potential properties, including an increase in width and a decrease in voltage threshold through pathways that remain to be elucidated.

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