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CCR2 Receptor Ligands Inhibit $Ca_v3.2$ T-Type Calcium Channels

Haitao You, Christophe Altier, and Gerald W Zamponi

Department of Physiology and Pharmacology, Hotchkiss Brain Institute, University of Calgary, Calgary, Canada

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ABSTRACT

Monocyte chemoattractant protein-1 (MCP-1) is a cytokine known to be involved in the recruitment of monocytes to sites of injury. MCP-1 activates the chemokine (C-C motif) receptor 2 (CCR2), a seven-transmembrane helix G protein-coupled receptor that has been implicated in inflammatory pain responses. Here we show that MCP-1 mediates activation of the CCR2 receptor and inhibits coexpressed N-type calcium channels in tsA-201 cells via a voltage-dependent pathway. Moreover, MCP-1 inhibits $Ca_v3.2$ calcium channels, but not other members of the Cav3 calcium channel family, with nanomolar affinity. Unlike in N-type channels, this modulation does not require CCR2 receptor activation and seems to involve a direct action of the ligand on the channel. Whole-cell T-type calcium currents in acutely dissociated dorsal root ganglia neurons are effectively inhibited by MCP-1, consistent with the notion that these cells express $Ca_v3.2$. The effects of MCP-1 were eliminated by heat denaturation. Furthermore, they were sensitive to the application of the divalent metal ion chelator diethylenetriaminepentaacetic acid, suggesting the possibility that metal ions may act as a cofactor. Finally, small organic CCR2 receptor antagonists inhibit $Ca_v3.2$ and other members of the T-type channel family with micromolar affinity. Our findings provide novel avenues for the design of small organic inhibitors of T-type calcium channels for the treatment of pain and other T-type channel linked disorders.

N- and T-type calcium channels are key mediators of nociceptive signaling in primary afferent pain fibers (Altier and Zamponi, 2004). T-type calcium channels are expressed in nerve endings of pain-sensing neurons, where they regulate neuronal excitability (Todorovic and Jevtovic-Todorovic, 2007). N-type calcium channels, on the other hand, are expressed at presynaptic nerve terminals in laminae I and II of the dorsal horn of the spinal cord, where they regulate the release of neurotransmitters such as substance P and glutamate and thus the communication between the primary afferent fibers and neurons projecting to the brain (for review, see Zamponi et al., 2009). Consequently, inhibition of either N- or T-type calcium channels has been shown to mediate analgesia. For example, intrathecal injection of the T-type channel inhibitor ethosuximide (Matthews and Dickenson, 2001) or antisense depletion of $Ca_v3.2$ T-type calcium channels (Bourinet et al., 2005) mediates potent analgesia in rodents. Likewise, gene knockout or in vivo knockdown of specific N-type channel splice isoforms (Saegusa et al., 2001; Altier et al., 2007), or their direct inhibition by peptide toxins isolated from fish-hunting cone snails (Staats et al., 2004) depresses pain signaling. Besides direct block by peptidergic or small organic calcium-channel blockers, voltage-gated calcium channels can also be inhibited by activation of certain types of G protein-coupled receptors, such as μ-opioid receptors, which reduce N-type calcium channel activity via a voltage-dependent pathway that involves binding of $G\beta\gamma$ subunits to the channel protein (for review, see Tedford and Zamponi, 2006).

Although activation of some types of receptors proves beneficial with regard to treating pain, other types of G protein-coupled receptors are pronociceptive (Negri et al., 2006). One such example is CCR2, a chemokine receptor that is linked to $G\alpha_{i/o}$. Its ligand, monocyte chemoattractant protein-1 (MCP-1; also known as CCL2), is a cytokine that is involved in the recruitment of monocytes to sites of injury (White et al., 2005a; Zhang and De Koninck, 2006). MCP-1 is ex-

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ABBREVIATIONS: CCR2, chemokine (C-C motif) receptor 2; MCP-1, monocyte chemoattractant protein-1; BMS CCR2 22, 2-[(isopropylaminocarbonyl)amino]-*N*-[2-[[cis-2-[[4-(methylthio)benzoyl]amino]cyclohexyl]amino]-2-oxoethyl]-5-(trifluoromethyl)benzamide; RS102895, 1′-[2-[4-(trifluoromethyl)phenyl]ethyl]-spiro[4*H*-3,1-*be* nzoxazine-4,4′-piperidin]-2(1*H*)-one; RS504393, 6-methyl-1′-[2-(5-methyl-2-phenyl-4-oxazolyl)ethyl]-spiro[4*H*-3,1-benzoxazine-4,4′-piperidin]-2(1*H*)-one; EYFP, enhanced yellow fluorescent protein; GDPβS, guanosine 5′-[β-thio]diphosphate; DTPA, diethylenetriaminepentaacetic acid; DRG, dorsal root ganglion.

pressed in sensory neurons, and its levels are up-regulated during nerve injury (Tanaka et al., 2004; White et al., 2005b). Its release may increase the excitability of surrounding neurons within the dorsal root ganglia, thereby resulting in proalgesia (White et al., 2007). This may be further exacerbated by release of MCP-1 from nerve terminals of primary afferent fibers, which has been shown to activate microglia in the spinal cord (Zhang et al., 2007; Thacker et al., 2009). MCP-1 is also expressed in astrocytes of the CNS and has been implicated in pain sensitization at the central level (Gao et al., 2009). Consistent with a proalgesic action of MCP-1, knockout of CCR2 receptors reduces inflammatory pain and prevents the occurrence of neuropathic pain as a result of chronic construction injury (Abbadie et al., 2003). Likewise, intrathecal administration of CCR2 receptor antagonists alleviates neuropathic pain (Dansereau et al., 2008; Abbadie et al., 2009). Given these proalgesic actions of MCP-1, we wondered whether they might in part arise from an enhancement of activation of N- or T-type calcium channel activity. As we show here, the exact opposite is the case as MCP-1 inhibits N-type calcium channels via a G protein-coupled pathway, whereas T-type calcium channels of the $Ca_v3.2$ subtype are inhibited directly and with high affinity through a receptor-independent pathway. Moreover, $Ca_v3.2$ channels can be inhibited by CCR2 receptor antagonists, which could thus serve as initial structural scaffolds for the development of higher affinity T-type channel blockers.

Materials and Methods

CDNA Constructs. Human $Ca_v3.2$, rat $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$, and $Ca_v2.3$ $\alpha1$ subunits, and ancillary rat $Ca_v\beta1b$ and $\alpha2$-$\delta1$ subunits were kindly provided by Dr. Terrance Snutch (University of British Columbia, Vancouver, BC, Canada). Human $Ca_v3.3$ was obtained from Dr. Arnaud Monteil (Universités Montpellier I & II, Montpellier, France), and human $Ca_v3.1$ was as we described previously (Beedle et al., 2002). Human CCR2a receptor cDNA was purchased from Missouri S&T cDNA Resource Center (Rolla, MO). The construction of $G\beta\gamma$ binding peptide βark-ct encoding cDNA in pIRES2-EGFP expression vector (Clontech, Mountain View, CA) was described previously (Magga et al., 2000).

Chemicals. Unless stated otherwise, chemicals and drugs were purchased from Sigma (St. Louis, MO). MCP-1 was purchased from R&D Systems (Minneapolis, MN) and BMS CCR2 22, RS102895 hydrochloride, and RS504393 were purchased from Tocris Bioscience (Ellisville, MO). Drugs were dissolved in DMSO at the following stock concentrations: 10 mM BMS CCR2 22, 50 mM RS102895 hydrochloride, and 10 mM RS504393. Calcium channel currents were not affected by 0.1% dimethyl sulfoxide.

tsA-201 and COS-7 Cell Culture and Transfection. Human embryonic kidney tsA-201 cells were cultured and transfected using the calcium phosphate method as described previously (Altier et al., 2006). Enhanced yellow fluorescent protein (EYFP) DNA (0.5 μg of pEYFP; Clontech) was transfected as a marker in experiments lacking an alternative fluorescent construct. For experiments involving L-, P/Q-, N-, and R-type calcium channels, $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$, and $Ca_v2.3$ calcium channel $\alpha1$ subunits (3 μg), respectively, were each cotransfected with rat $\beta1b$ (3 μg) and rat $\alpha2$-$\delta1$ (3 μg). For experiments involving T-type calcium channels, $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ $\alpha1$ subunits (6 μg) were transfected individually. For experiments involving $G\beta\gamma$ binding peptide βark-ct in pIRES2-EGFP, cells were transfected as above, but cDNA encoding the peptide (3 μg) was transfected in place of the EYFP marker. African green monkey kidney COS-7 cells were cultured using the same method as tsA-201 cells. COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h. Cells were resuspended with 0.25% (w/v) trypsin-EDTA (Invitrogen) and plated on glass coverslips 3 to 4 h before patching.

DRG Neurons. Dorsal root ganglion (DRG) neurons from rats were acutely isolated as described previously (Beedle et al., 2004). In brief, Sprague-Dawley rats (30–40 days old) were anesthetized with halothane and euthanized in accordance with the University of Calgary Animal Care and Use Policy. The DRGs from all spinal levels were collected in Ham's F12 medium (Invitrogen) supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). DRG neurons were dissociated using Collagenase A (Invitrogen) for 40 min at 37°C followed by six to eight times trituration in the medium through a reduced fire-polished Pasteur pipette tip. The resulting suspension was plated on coverslips coated with 20 μg/ml poly-L-lysine (Sigma, St. Louis, MO) and kept at 37°C and 5% CO_2. Patch-clamp recordings were carried out at room temperature 2 to 24 h after plating.

Electrophysiology. Whole-cell voltage-clamp recording for tsA-201 and COS-7 cells were performed 2 to 4 days after the transfection. The external recording solution contained 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM TEA-Cl, 65 mM CsCl, and 10 mM *d*-glucose, pH adjusted to 7.2 with TEA-OH. The external recording solution for DRG neurons was composed of 2 mM $CaCl_2$, 160 mM TEA-Cl, 10 mM HEPES, and 10 mM *d*-glucose, pH adjusted to 7.4 with TEA-OH. The external solution for DRG neuron recordings was supplemented with 1 μM nifedipine. The internal pipette solution was composed of 108 mM Cs-methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, and 9 mM HEPES, pH adjusted to 7.2 with CsOH. The internal solution was supplemented with 0.6 mM GTP and 2 mM ATP, which were added directly to the internal solution immediately before use. In some experiments, the GTP/ATP supplement was excluded or replaced with 1 mM guanosine 5′-[β-thio]diphosphate (GDPβS) as indicated. We know from our previous work that this is an effective means of interfering with receptor-mediated inhibition of T-type calcium channels (Iftinca et al., 2007). Drugs were prepared daily in external solution and were applied locally to cells with the use of a custom microperfusion system that allows solution exchanges in approximately 1 s (Feng et al., 2003). Currents were elicited from a holding potential of −100 mV and were measured by conventional whole-cell patch clamp using an Axopatch 200B amplifier in combination with Clampex 9.2 software (Molecular Devices, Sunnyvale, CA). Data were filtered at 1 kHz (8-pole Bessel) and digitized at 10 kHz with a Digidata 1320 (Molecular Devices). An online leak-subtraction protocol was used in which four pulses of opposite polarity and one-quarter amplitude were acquired immediately before the test protocol. We have described the prepulse facilitation protocol used in N- and L-type calcium channel studies previously (Kisilevsky et al., 2008). The extent of $G\beta\gamma$-mediated voltage-dependent inhibition was measured as the amount of prepulse relief, and the voltage-independent inhibition was calculated as the amount of inhibition remaining. For current-voltage relationship studies, the membrane potential was held at −100 mV and was depolarized from −90 to +50 mV with 10-mV increments. For steady-state inactivation studies, the membrane potential was depolarized by test pulses to −20 mV after 3.6-s conditioning prepulses ranging from −110 to 0 mV. The current amplitude obtained from each test pulse was then normalized to the maximum at −110 mV.

Data Analysis and Statistics. Data were analyzed using Clampfit 9.2 (Molecular Devices) and Prism 5 software (GraphPad Software Inc., San Diego, CA). Because MCP-1 incompletely inhibited $Ca_v3.2$ channel activity, concentration-response curves for MCP-1 inhibition were analyzed using a modified Hill equation: $I/I_{control} = C + (1 - C)(1/(1 + ([\text{MCP-1}]/\text{IC}_{50})^{n_H}))$, where C reflects the fraction of current that remains at saturating MCP-1 concentrations and n_H is the Hill coefficient. Steady-state inactivation curves were fitted using the Boltzmann equation: $I = 1/(1 + e^{(V - V_h)/k})$, where V_h is the half-inactivating potential and k is the slope factor. Current-voltage plots were fitted using the modified Boltzmann equation:

$I = 1/(1 + e^{-(V - V_a)/k}) \times G \times (V - E_{rev})$, where E_{rev} is the reversal potential, G is the maximum slope conductance, k is a slope factor, and V_a is the half-activation potential. The time courses of development of block and washout at various MCP-1 concentrations were fitted with monoexponential equations.

Statistical analysis was performed using paired or unpaired t-tests as appropriate. Statistical significance was set at $P < 0.05$, and all error bars represent S.E.M.

Results

CCR2 Receptor Activation Mediates Voltage-Dependent Inhibition of N-Type Calcium Channels. To determine the effects of CCR2 receptor activation on voltage-gated calcium channels, initially one member of each of the major classes of calcium channels (i.e., Ca_v1, Ca_v2, and Ca_v3) was coexpressed in tsA-201 cells with cDNA encoding for the CCR2 receptor. In the case of high-voltage-activated channels, the ancillary β and $\alpha 2\delta$ subunits were coexpressed. The cells were then functionally assayed using whole-cell patch-clamp recordings and a double-pulse protocol in which a test pulse to +20 mV was followed by a strong depolarizing prepulse to +150 mV before application of a second test pulse to +20 mV. As shown in Fig. 1A, $Ca_v1.2$ (i.e., L-type) channels were insensitive to CCR2 receptor activation by 100 nM MCP-1. In contrast, N-type $Ca_v2.2$ channels underwent a pronounced receptor-mediated inhibition that could be almost completely reversed by the depolarizing prepulse, consistent with membrane delimited, G$\beta\gamma$-mediated inhibition (Tedford and Zamponi, 2006). $Ca_v3.2$ (T-type) calcium channels also underwent a pronounced MCP-1-induced inhibition. Figure 1B summarizes the inhibitory effects of MCP-1 on the three calcium channel subtypes, revealing an overall inhibition of 50% for $Ca_v2.2$ and $Ca_v3.2$ channels. Figure 1C examines the extent of prepulse relief from $Ca_v2.2$ channels in the absence and the presence of cotransfected CCR2 receptors. As seen from the figure, no prepulse relief occurred in cells lacking the CCR2 receptor, irrespective of agonist application. A small degree of prepulse relief was observed simply upon coexpression of the receptor in the absence of agonist and was significantly augmented after agonist application. Overall, the data obtained with $Ca_v2.2$ are consistent with those obtained with a wide range of $G\alpha_{i/o}$-linked receptors (Tedford and Zamponi, 2006). The observation that most of the inhibition was voltage-dependent (Fig. 1D), however, contrasts with findings obtained with other receptor types such as dopamine (Kisilevsky and Zamponi, 2008) or nociceptin receptors (Beedle et al., 2004).

$Ca_v3.2$ Channels Undergo Receptor Independent Inhibition by MCP-1. To determine whether the MCP-1 effects on $Ca_v2.2$ and $Ca_v3.2$ channels were mediated by CCR2 receptor activation, we applied 100 nM MCP-1 to cells lacking the receptor. As shown in Fig. 2B, $Ca_v2.2$ channels were not inhibited by MCP-1 in the absence of a coexpressed CCR2 receptor. These data confirm the involvement of a CCR2 receptor in the inhibition of N-type channel activity; furthermore, they indicate that tsA-201 cells do not endogenously express significant levels of functional CCR2 receptors. It is noteworthy that MCP-1 was as effective in inhibiting $Ca_v3.2$ channel activity in the absence of CCR2 (Fig. 2, A and B) as in its presence (Fig. 1, A and B). In contrast, neither $Ca_v3.1$ nor $Ca_v3.3$ channels were inhibited by MCP-1 (Fig. 2, A and B), and there was no effect on $Ca_v2.1$ (P/Q-type) and $Ca_v2.3$ (R-type) channels. The inhibitory effects of MCP-1 on $Ca_v3.2$ channels were not a specific feature of tsA-201 cells, because a similar degree of inhibition was observed for $Ca_v3.2$ channels expressed in COS-7 cells (Fig. 2B). The effects of MCP-1 on transiently expressed $Ca_v3.2$ channels were maintained upon inclusion of GDPβS in the patch pipette and after removal of intracellular GTP and ATP (Fig. 2C). Finally, coexpression of the G$\beta\gamma$ binding peptide βark-ct, which we

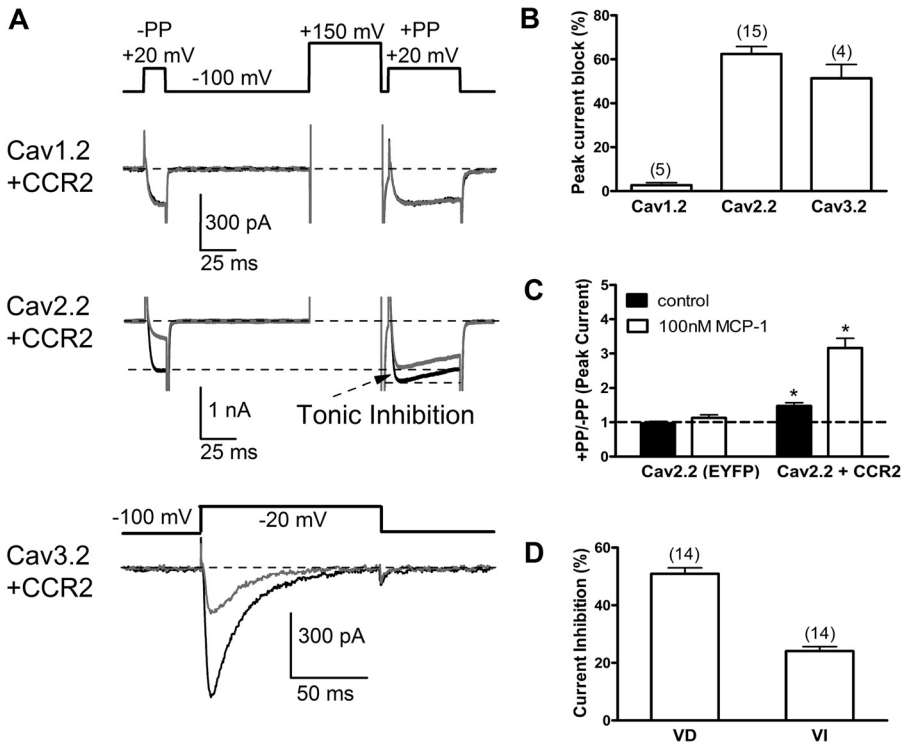

Fig. 1. Effect of 100 nM MCP-1 on $Ca_v1.2$, $Ca_v2.2$, and $Ca_v3.2$ calcium channels coexpressed with CCR2 in tsA-201 cells. A, currents obtained from transiently expressed $Ca_v1.2$ (top), $Ca_v2.2$ (middle), and $Ca_v3.2$ (bottom) channels in the absence (black traces) and presence (gray traces) of MCP-1 (100 nM), respectively. For $Ca_v1.2$ and $Ca_v2.2$ calcium channels, a prepulse protocol was used to determine the degree of tonic inhibition (without activation of CCR2) and voltage-dependent and -independent inhibition after activation of CCR2 (see *Materials and Methods*). For $Ca_v3.2$ channels, no prepulse protocol was used because of voltage dependent channel inactivation during the prepulse. Instead, a single test depolarization to −20 mV was applied. B, peak current block elicited by application of 100 nM MCP-1 for $Ca_v1.2$, $Ca_v2.2$, and $Ca_v3.2$ calcium channels coexpressed with CCR2. C, prepulse relief from CCR2 modulation of $Ca_v2.2$ channels in the presence and absence of MCP-1. D, relative magnitude of voltage-dependent (VD) and voltage-independent (VI) modulation of $Ca_v2.2$ channels by MCP-1. Error bars represent S.E.; numbers in parentheses reflect numbers of experiments. Asterisks indicate statistical significance relative to control conditions.

have shown previously to prevent Gβγ modulation of N-type channels (Magga et al., 2000), did not affect MCP-1 inhibition of $Ca_v3.2$ channels (Fig. 2C). This indicates that $Ca_v3.2$ channel activity is not inhibited via the Gβγ pathway described previously (Wolfe et al., 2003; DePuy et al., 2006). Taken together, these data indicate that MCP-1 inhibits $Ca_v3.2$ channels via a receptor-independent pathway that may involve a direct inhibition of channel activity by MCP-1.

Biophysical Characterization of MCP-1 Block of $Ca_v3.2$ Channels. Application of MCP-1 to $Ca_v3.2$ channels did not affect the voltage dependence of activation, as is evident from the whole-cell current waveforms shown in Fig. 3A and from the ensemble current-voltage relationships in Fig. 3B. These data indicate that MCP-1 does not act as a gating inhibitor as observed with peptides such as kurtoxin (Sidach and Mintz, 2002). In contrast, there was a statistically significant hyperpolarizing shift in the midpoint of the steady-state inactivation curve of the channel by approximately 6 mV, which would be expected to produce further inhibitory effects at a normal neuronal resting potential (Fig. 3C). Examination of the dose dependence of MCP-1 action (Fig. 3D) reveals that MCP-1 is a partial inhibitor of $Ca_v3.2$ with a maximum inhibition of approximately 50% at 100 nM MCP-1. The concentration required to reach half of this maximal inhibition (referred here as IC_{50}) was 5.6 nM, suggesting a relatively high-affinity interaction between the channel and the MCP-1 molecule. A kinetic analysis of the time course of development of block and washout at various MCP-1 concentrations revealed only a weak linear dependence of the inverse of the time constant for development of block on MCP-1 concentration (Fig. 3, E and F). Assuming a 1:1 blocking interaction, the slope of the regression line and intercept on the ordinate would suggest blocking (k_{on}) and unblocking (k_{off}) rate constants of, respectively, 3.29×10^{-4} nM^{-1} s^{-1} and 0.040 s^{-1}. Although the latter value is similar to that obtained from the washout of the MCP-1 effect ($k_{off} = 0.048 \pm 0.012$ s^{-1}, $n = 6$), the ratio of unblocking to blocking rate constant obtained from the regression analysis yields an equilibrium dissociation constant of 121 nM, which is approximately 20-fold larger than the value obtained from the dose-response curve shown in Fig. 3D. Hence, our data are inconsistent with a simple 1:1 interaction between MCP-1 and the channel, suggesting a more complex action of MCP-1 on the channel, perhaps at more than one site.

$Ca_v3.2$ channels are known to be potently inhibited by divalent metal ions, such as zinc (Traboulsie at el., 2007), and to a much greater extent than other members of the T-type

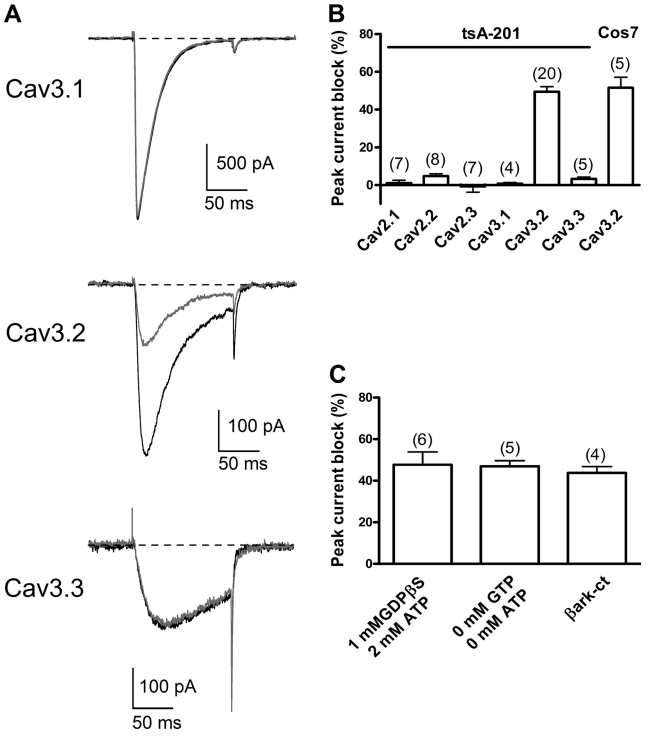

Fig. 2. Effect of 100 nM MCP-1 on Ca_v2 and Ca_v3 channels in the absence of coexpressed CCR2. A, representative current traces for MCP-1 inhibition of $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ T-type calcium channels. B, peak current block induced by application of 100 nM MCP-1 of various channels expressed in tsA-201 cells and of $Ca_v3.2$ expressed in Cos7 cells. C, effect of GDPβS, removal of intracellular GTP and ATP, or coexpression of the βark-ct on MCP-1 inhibition of $Ca_v3.2$. Error bars represent S.E.; numbers in parentheses reflect numbers of experiments.

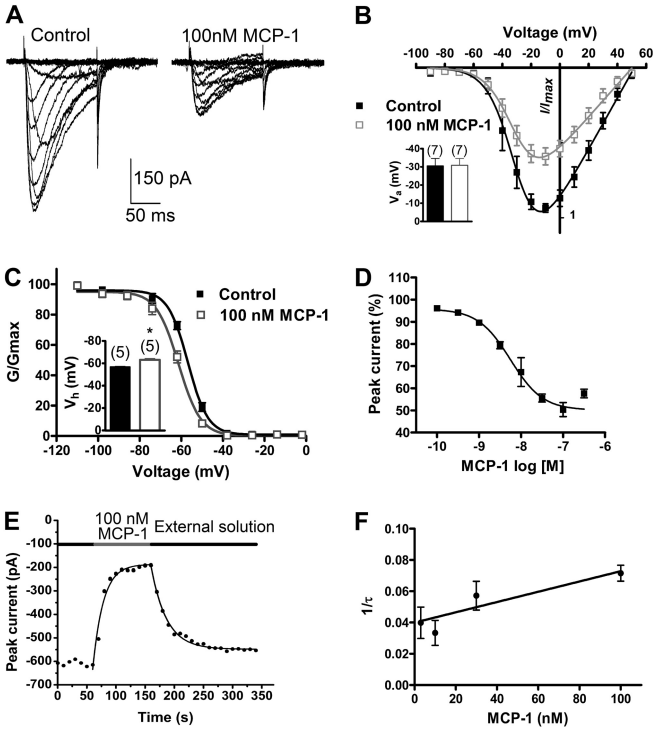

Fig. 3. Biophysical properties of MCP-1 block of $Ca_v3.2$ channels. A, representative families of whole cell currents of $Ca_v3.2$ in the absence and presence of 100 nM MCP-1. The current traces were elicited by a series of step depolarizations from a holding potential of −100 mV. B, normalized current-voltage relations of $Ca_v3.2$ channels before and after application of 100 nM MCP-1. The half-activation potentials were −30.5 ± 4.1 mV and −30.8 ± 3.7 mV before and after application of MCP-1, respectively (inset, $P > 0.05$, paired t test). C, steady-state inactivation curves obtained from $Ca_v3.2$ channels before and after application of MCP-1. The half-inactivation potentials were −56.9 ± 0.5 mV and −63.1 ± 1.2 mV before and after the treatment with MCP-1, respectively (inset, $P < 0.05$, paired t test). D, dose dependence of MCP-1 inhibition of the $Ca_v3.2$ peak current amplitude. The data were fitted with a modified Hill equation that accounts for partial block. The half-maximal inhibitory concentration from the fit was 5.6 ± 1.4 nM and the Hill coefficient was 1.12 ($n = 3$~7 for each concentration point). E, representative time course of the development of and recovery from MCP-1 block. The time courses of inhibition and washout were fitted monoexponentially. F, the inverse of the time constant for development of block as a function of MCP-1 concentration. The slope of the regression is 3.29×10^{-4} nM^{-1} s^{-1} and the y-intercept is 4.0×10^{-2} s^{-1}. Error bars represent S.E.; numbers in parentheses reflect numbers of experiments.

channel family. To rule out the possibility that the selective inhibition of $Ca_v3.2$ channels by MCP-1 might be mediated by trace amount of metal ions in the MCP-1 sample, we examined MCP-1 action in the presence of the divalent metal ion chelator DTPA (Nelson et al., 2007). Application of 200 μM DTPA resulted in an enhancement of $Ca_v3.2$ currents by 34.6% ($n = 5$), suggesting that a fraction of the $Ca_v3.2$ channels are tonically inhibited by metal ions. Curiously, subsequent application of MCP-1 in the continued presence of DTPA inhibited $Ca_v3.2$ channel activity only weakly (1.9% inhibition at 100 nM MCP-1, $n = 5$). This was not due to a direct effect of DTPA on MCP-1, because MCP-1-mediated inhibition of $Ca_v2.2$ channels by CCR2 receptors was unaffected by the chelator (56.7% inhibition, $n = 3$). Although this could suggest that the observed effects of MCP-1 on $Ca_v3.2$ channel activity might be due to contaminating metal ions, this is unlikely for two reasons. First, divalent metal ions such as zinc, copper, and nickel are known to mediate complete inhibition of $Ca_v3.2$ channel activity (see Traboulsie et al., 2007), whereas the effects of MCP-1 did not exceed approximately 50% inhibition, even at concentrations 60 times higher than the IC_{50}. Moreover, when MCP-1 was denatured by heating (100°C for 5 min), its inhibitory effects on $Ca_v3.2$ channels were largely abolished (2.3% inhibition at 100 nM, $n = 4$). Finally, we noted on two occasions that prolonged storage (2 months) of dissolved MCP-1 at −20°C resulted in a loss of channel-blocking activity, altogether supporting a specific action of MCP-1 on $Ca_v3.2$ rather than the involvement of a metal ion contaminant.

MCP-1 Blocks Native T-Type Currents. To rule out expression system artifacts and to determine whether native $Ca_v3.2$ channels are subject to MCP-1inhibition, we examined the effects of MCP-1 on native T-type currents in acutely dissociated rat DRG neurons. These cells express large T-type currents that are carried predominantly by $Ca_v3.2$ (Bourinet et al., 2005, Todorovic and Jevtovic-Todorovic, 2008). As shown in Fig. 4, application of 100 nM MCP-1 mediated a reversible inhibition of native T-type currents to an extent similar to that observed in tsA-201 cells. Hence, $Ca_v3.2$ channel inhibition by MCP-1 is readily observed in native cells and, in particular, in a cell type relevant to the transmission of pain signals.

MCP-1 Receptor Antagonists Inhibit $Ca_v3.2$ Channels. The effects of MCP-1 on $Ca_v3.2$ channels suggest the existence of an MCP-1 binding site on the channel protein that may share some structural features with the ligand binding pocket on the CCR2 receptor. To examine this possibility, we tested the effects of three other CCR2 ligands on $Ca_v3.2$ channels transiently expressed in tsA-201 cells. There are several high-affinity small organic CCR2 receptor antagonists (Mirzadegan et al., 2000; Cherney et al., 2008), with binding affinities in the low to mid nanomolar range. At a concentration of 10 μM, all of these compounds inhibited transiently expressed $Ca_v3.2$ channels. A more detailed analysis for RS504393 revealed that $Ca_v3.2$ channels are inhibited in a concentration-dependent manner, with greater than 90% inhibition at 30 μM, in contrast with the partial block seen with MCP-1. For transiently expressed $Ca_v3.2$ channels, a ~10-mV hyperpolarizing shift in the steady-state inactivation curve was observed after application of 10 μM RS504393 (Fig. 5B), consistent with a higher affinity of this compound for inactivated channels. RS504393 also inhibited native T-type currents in DRG neurons (Fig. 5C). Unlike the selective effects of MCP-1 on $Ca_v3.2$, RS504393 inhibited a wide range of voltage gated calcium channels, including other members of the Ca_v3 channel family, as well as N-, P/Q-, L-, and R-type channels. Taken together, these data indicate that several types of CCR2 receptor antagonists have the propensity to block voltage-gated calcium, albeit at concentrations higher than those required for CCR2 receptor inhibition.

Discussion

Possible Mechanism of Action of MCP-1. Our data show that in addition to inhibiting N-type channels via activation of CCR2 receptors, MCP-1 also inhibits $Ca_v3.2$ calcium channels independently of G protein activation. In principle, it may be possible that the channels could be inhibited via a G protein-independent "transinactivation" mechanism mediated by endogenously expressed receptors; however, this is unlikely considering our observations that endogenous CCR2 receptor levels are sufficiently low to preclude receptor-mediated inhibition of N-type channels in the same cell system. Hence, the most likely mechanism involves a direct interaction between MCP-1 and the $Ca_v3.2$ channel. MCP-1 did not affect the half-activation potential of the channel; hence, we can rule out the possibility that MCP-1 is a gating inhibitor similar to kurtoxin (Sidach and Mintz, 2002). It is also unlikely that MCP-1 reduces the numbers of channels in the plasma membrane, because the effects of MCP-1 had a fast rate of onset and were rapidly reversible upon washout. The observation that MCP-1 mediated only partial block of $Ca_v3.2$ could be due to either an allosteric effect of MCP-1 on channel activity (for example, a reduction of the maximal open probability), or partial occlusion of the pore, as has been reported for certain types of large calcium channel-blocking peptide toxins (Mintz, 1994), and thus a reduced single-

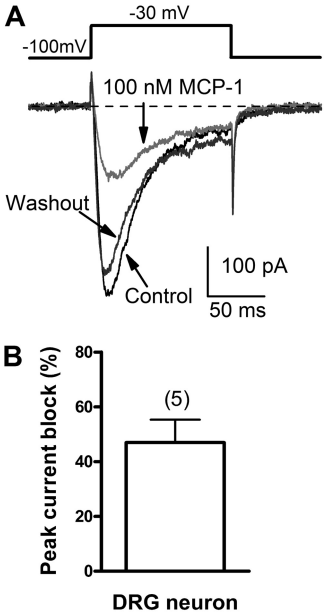

Fig. 4. MCP-1 blocks native T-type currents. A, representative traces of T-type currents obtained from acutely dissociated rat DRG neurons showing the effect of application of 100 nM MCP-1 and the washout. The holding potential was −100 mV, and the current was elicited by a depolarizing step to −30 mV. B, summary of peak current block of native T-type channels by 100 nM MCP-1.

channel conductance. Although partial pore block can account for the general observations with MCP-1, the detailed kinetic analysis of the time course of development of block argues against a simple 1:1 blocking interaction between the channel and the MCP-1 molecule. Future experiments examining the effects of known T-type channel inhibitors on MCP-1-bound channels, and permeation studies on channels partially inhibited by MCP-1, may provide further insights into the mechanism of MCP-1 action.

The observation that the metal ion chelator DTPA prevented the actions of MCP-1 suggests that the presence of metal ions is required for MCP-1 to exert its effects. This is reminiscent of observations by Nelson and colleagues (2007), who showed that the selective inhibitory actions of ascorbate on $Ca_v3.2$ channel activity required the presence of zinc ions that are present at trace amounts in the external recording solution, although the actions of ascorbate are likely distinct from those of MCP-1. $Ca_v3.2$ channels contain a unique histidine residue in position 191 that functionally interacts with metal ions such as zinc and nickel (Kang et al., 2006; Nelson et al., 2007). It remains to be determined whether this residue is involved in the actions of MCP-1.

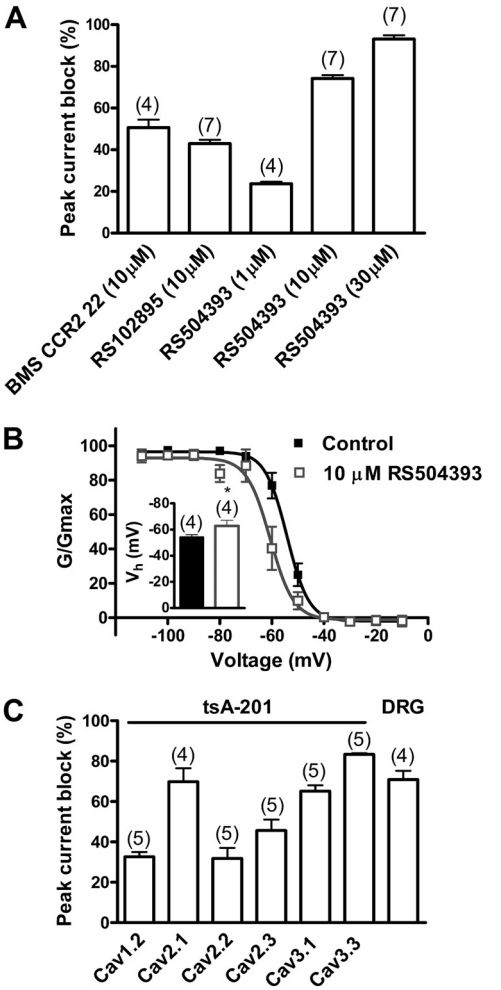

Fig. 5. Inhibitory effects of CCR2 antagonists on transiently expressed and native calcium channels. A, summary of peak current block of $Ca_v3.2$ channel by 10 μM BMS CCR2 22, RS102895, and RS504393. B, steady-state inactivation curves obtained from $Ca_v3.2$ channels before and after application of 10 μM RS504393. The half-inactivation potentials were -54.2 ± 1.8 and -62.7 ± 4.3 mV before and after the treatment with RS504393, respectively (inset, $P < 0.05$, paired t test). C, inhibition of native T-type calcium channels in DRG neurons and of various voltage-gated calcium channels expressed in tsA-201 cells by 10 μM RS504393. Error bars represent S.E.; numbers in parentheses reflect numbers of experiments.

Mixed CCR2/T-Type Channel Antagonists—A New Approach to Treating Pain? Our data reveal an unexpected action of CCR2 receptor ligands on both native and transiently expressed T-type calcium channels. Both CCR2 receptors and T-type channels have a pronociceptive function. Knockout of CCR 2 receptors results in a phenotype that is hyposensitive to pain (Abbadie et al., 2003), and intrathecal delivery of CCR2 receptor antagonists is beneficial in the treatment of neuropathic pain (Dansereau et al., 2008; Abbadie et al., 2009). Either short-term block or antisense depletion of $Ca_v3.2$ T-type calcium channels also mediates analgesia (Matthews and Dickinson, 2001; Bourinet et al., 2005). Our experiments indicate that CCR2 receptors do not directly modulate T-type calcium channels (i.e., all of the observed MCP-1 effects on T-type channel function occurred independently of the receptors). One might therefore expect that dual CCR2/$Ca_v3.2$ channel antagonists would have additive effects in blocking pain behavior. In this context, it will be interesting to determine the effect of compounds such as RS504393 in animal models of pain. A recent study has reported analgesic effects of a novel proprietary CCR2 receptor antagonist (Jung et al., 2009). At this point, it remains to be determined whether the analgesic actions of this compound are due purely to CCR2 receptor antagonist activity or if inhibition of $Ca_v3.2$ channels might also be involved.

A number of compounds with diverse chemical structures have been shown to inhibit T-type calcium channels (Belardetti and Zamponi, 2008). The chemical structures of the CCR2 receptor antagonists investigated here diverge from known T-type channel-blocking pharmacophores. These CCR2 receptor antagonists could potentially serve as novel scaffolds for the development of higher affinity (i.e., in the mid nanomolar range, as seen with CCR2 receptor affinity) T-type channel inhibitors, but with increased selectivity for $Ca_v3.2$ over other calcium channel isoforms. If CCR2 receptor antagonist activity can be maintained, then this might indeed provide the potential for novel and highly efficacious analgesics with a dual mode of action. Finally, it is worth noting that CCR2 receptor activation resulted in inhibition of N-type calcium channels in tsA-201 cells. Although the receptors are clearly capable of inhibiting N-type channel activity under conditions in which they are artificially coexpressed, it remains to be determined whether such a regulation can occur in intact presynaptic nerve terminals in the dorsal horn of the spinal cord. Indeed, there is recent evidence that CCR2 activation occurs both at sites of injury and in the dorsal root ganglia, but not in the spinal cord (Jung et al., 2009), where N-type channels control neurotransmitter release from primary afferent fibers. The notion that CCR2 receptor activation is associated with pronociceptive effects (Dansereau et al., 2008) rather than analgesia further suggests that N-type calcium channels in the dorsal horn of the spinal cord may not be physiological targets of CCR2 receptor activation.

Overall, our data reveal a novel action of the cytokine MCP-1 in regulating T-type calcium channel activity though direct inhibition. The existence of binding site on $Ca_v3.2$ that

can interact with CCR2 receptor ligands may provide for a substrate for the development of novel analgesics.

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Address correspondence to: Dr. Gerald W. Zamponi, Department of Physiology and Pharmacology, University of Calgary, 3330 Hospital Dr. NW, Calgary, AB, T2N 4N1, Canada. E-mail: zamponi@ucalgary.ca