Mineralocorticoid Receptor Blocker Eplerenone Reduces Pain Behaviors In Vivo and Decreases Excitability in Small-diameter Sensory Neurons from Local Inflamed Dorsal Root Ganglia In Vitro

Fei Dong, M.D.,* Wenrui Xie, Ph.D.,† Judith A. Strong, Ph.D.,‡ Jun-Ming Zhang, M.D., M.Sc.§

ABSTRACT

Background: Inflammation of the dorsal root ganglia (DRG) may contribute to low back pain, postherpetic neuralgia, and neuropathic pain. The mineralocorticoid receptor (MR) plays a proinflammatory role in many nonrenal tissues, but its role in peripheral pain at the DRG level is not well studied.

Methods: Local inflammation of the L5 DRG with the immune activator zymosan rapidly leads to mechanical hypersensitivity and increased excitability of sensory neurons. Using this pain model, the authors applied the MR antagonist eplerenone locally to the inflamed DRG. Excitability of small-diameter sensory neurons was examined in acute primary culture by using patch clamp techniques.

Results: Local eplerenone significantly reduced the mechanical hypersensitivity and shortened its duration. The same dose was ineffective systemically. Immunohistochemical studies showed the MR was present in most neurons and rapidly translocated to the nucleus 1 day after local DRG inflammation. Activation of satellite glia (defined by expression of glial fibrillary acidic protein) in the inflamed DRG was also reduced by local eplerenone. Increased excitability of small-diameter sensory neurons 1 day after inflammation could be observed in vitro. Eplerenone applied in vitro (8–12 h) could reverse this increased excitability. Eplerenone had no effect in neurons isolated from normal, uninflamed DRG. The MR agonist aldosterone (10 nM) applied in vitro increased excitability of neurons isolated from normal DRG.

Conclusions: The MR may have a pronociceptive role in the DRG. Some of its effects may be mediated by neuronal MR. The MR may represent a novel therapeutic target in some pain syndromes.

OW back pain is an extremely common problem that remains difficult to treat.1,2 Although the underlying mechanisms are still not well understood, inflammatory irritation of lumbar dorsal root ganglion (DRG), either from direct chemical irritation or secondary to an immune response to the nucleus pulposus, may contribute to low back pain.3,4 Moreover, there is partial effectiveness of antiinflammatory drugs in treating pain associated with inflammation.

This article is accompanied by an Editorial View. Please see: Schumacher MA: Back pain and the mineralocorticoid receptor: Is there a connection? Anesthesiology 2012; 117:951–2.
The mineralocorticoid receptor (MR) was originally viewed only as the target of aldosterone, promoting Na+ and K+ transport in epithelial cells, best known for its role in the kidney and colon. More recently, the MR has been detected in nonepithelial tissues, notably in the heart and brain. Corticosterone (in rodents; cortisol in humans), and some steroid medications can activate not only the glucocorticoid receptor (GR) but also the MR; in most tissues, MR may be physiologically activated primarily by corticosterone, which has a much higher plasma concentration than aldosterone does. In the kidney, the MR is activated only by aldosterone due to the presence of enzymes that inactivate glucocorticoids. MR activation promotes M1 or classical inflammation (high levels of oxidative metabolites and proinflammatory cytokines, tissue destruction), whereas GR activation promotes M2 or alternative inflammation (tissue remodelling and wound repair). Recent evidence shows that inflammation is associated with MR activation in kidney, heart, and central nervous system. Further, blockers of MR not only suppress the inflammatory reaction but also promote wound healing and functional recovery after nerve injury (for review). Some of these studies used the new MR antagonist eplerenone, which has much better selectivity for MR over GR than previous agents did. However, the significance of MR activation in pathological pain remains elusive, and there are few studies regarding its role in peripheral sensory ganglia. In the present study, we examined the existence and localization of MR in the DRG in normal and locally inflamed DRG. We also examined effects of eplerenone on pain behavior and excitability of sensory neurons. We used our recently developed rat model in which long-lasting pain is induced by locally inflaming sensory ganglia by depositing a drop of the immune activator zymosan over a single lumbar DRG. This results in prolonged mechanical pain, rapid upregulation of proinflammatory cytokines, and increased sensory neuron excitability. Excitatory effects on small diameter, presumptive nociceptors could be preserved in short-term culture. We took advantage of this finding to study effects of eplerenone in vitro, where effects on neurons could be examined independently of possible in vivo eplerenone effects on immune or other cells.

Materials and Methods

Animals
All the surgical procedures and the experimental protocol were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati (Cincinnati, Ohio). All experiments adhered to the guidelines laid out in the Guide for the Care and Use of Laboratory Animals. Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were used for all behavioral experiments. Rats were housed one or two per cage under a controlled diurnal cycle of 12-h light and 12-h dark with free access to water and food. The ambient environment was maintained at constant temperature (22 ± 0.5°C) and relative humidity (60–70%).

Surgical Procedure for Localized DRG Inflammation

The surgery was performed as previously described. Male Sprague-Dawley rats weighing 200–250 g (for behavioral and immunohistochemical experiments) or female Sprague-Dawley rats weighing 80–100 g (for electrophysiological experiments) were anesthetized by isoflurane. Our previous results using this model showed that behavior changes in smaller female rats used for recording were similar to those in large male rats used for behavior. An incision was made along the spine from S1 to L4 vertebral level. For behavioral experiments, the L5 intervertebral foramen was visualized by exposing L5 and L4 transverse processes by separating the overlying back spine paraspinal muscles. The immune activator zymosan (2 mg/ml, 10 µl, in incomplete Freund adjuvant (IFA) was injected beneath the L5 intervertebral foramen, above the DRG, via a needle (30-G1/2”) that was bent into a 90° angle 1–2 mm from the tip. During injection, the bent part of needle was inserted into the intervertebral foramen and kept there for 1–2 min after injection to avoid leakage. With the same methods, both L4 and L5 DRG were inflamed for electrophysiological and immunostaining experiments. Eplerenone and other steroids are very insoluble in water. In order to apply eplerenone (Tocris, Bristol, United Kingdom) locally to the inflamed DRG, 500 µg of the drug was added to the oily IFA + zymosan used to inflame the L5 DRG (Zym + Epl group). Cholesterol was used as a chemically similar negative control (Zym + Cho group). The method was chosen based on a published study, in which steroid micropellets implanted into the brain had a diffusion radius of approximately 750 µm and a duration of action of 5–7 days. In that study, aldosterone pellets in brain had behavioral effects at 15 and 30 µg but not at 3 µg; however, we chose to use 500 µg eplerenone in our study because eplerenone, though specific for MR over GR and other steroid receptors, has relatively low affinity for the MR (half maximal inhibitory concentration several orders of magnitude higher than the half maximal effective concentration for aldosterone or corticosterone). In some experiments, to control for possible systemic effects of eplerenone, the same amount was implanted subcutaneously under the skin of the back (Zym + Epl [s.c.]).

Behavioral Testing

Animals were inspected and tested every other day for three trials before local inflammation of the DRG surgery (baseline) and after surgery as indicated. Mechanical sensitivity was tested by applying a series of von Frey filaments to the heel region of the paw, using the up-and-down method. A cutoff value of 15 g was assigned to animals that did not respond to the highest filament strength used. Rearing behavior in a novel environment was measured by taping animal behavior immediately after placing the animal in a 15.5” × 15.5” chamber, during the
daytime but under dim red light illumination. Taping was done under the experimenter absent from the room. The incidence and duration of rears was scored offline.

**Quantitative Polymerase Chain Reaction**

Quantitative polymerase chain reaction methods were used as previously described. Primers designed with Primer-BLAST were chosen to anneal at 60°C, and to either sit on or amplify across an exon boundary to avoid amplifying genomic DNA. All amplicons were initially confirmed by agarose gel electrophoresis to determine whether the amplicon was the predicted size and a single product. Oligonucleotide primers used in this study were synthesized by Invitrogen (Carlsbad, CA). Primer sequences were: GR (Nr3c1; gene ID 24413): forward: AGCCGTACCTCCCTTGGGGGC; reverse: AGGTTGAGGGTGCTTCCGG. MR (Nr3c2; gene ID 25672): forward: GGAGAGTAGGTGATCCCCGT; reverse: ACCCCTAGTGACACCCAGGCC. hypoxanthine guanine phosphoribosyl transferase (HPRT; gene ID 24465): forward: GCAGACTTTGCTTTGCC; reverse: TACTGGCCACATCAACAGGA.

**Immunostaining of MR and Glial Fibrillary Acidic Protein in the DRG and Primary Culture**

Rats were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal) and perfused with 0.1 M phosphate buffer (pH = 7.4) followed by 200–300 ml of Zamboni fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH = 7.4) through the left ventricle of the heart. The inflamed L5/L4 DRG were removed, postfixed in the perfusion fixative for 30 min at 4°C and then transferred to 20% sucrose overnight. Frozen sections of 10 μm were cut from the harvested DRG. Rats were sacrificed between 5 PM and 7 PM. For cultured cells, coverslips (after patch clamp experiments) were put into −20°C acetone for 10 min. Sections or dried coverslips were permeabilized twice for 5 min in phosphate-buffered saline with 0.3% Triton X-100 (PBST), blocked for 1.5 h with 1% bovine serum albumin and 3% normal goat serum in PBST. After washing in PBST, sections were mounted on coverslips with Vector Hard Set mounting medium (Vector Laboratories, Burlingame, CA). Using SlideBook Digital Microscopy Software (Intelligent Imaging Innovation, Santa Monica, CA), confocal images from multiple sections were digitized under a light microscope equipped with a color digital camera and stored in a computer. Additional experiments using a different, monoclonal antibody to the MR (antibody 1D5 in reference 18) were conducted with the same protocol in sections from normal DRG, except that the dilution used was 1:200, and the primary antibody incubation medium contained 0.1% bovine serum albumin instead of normal goat serum. To quantitate the expression of MR in neurons, we counted at least six nonsequential sections from each single DRG, scoring neurons as expressing MR in cytoplasm, plasma membrane, and nucleus. The fractions of cells in each group were averaged for each animal, and statistical comparisons were done using the animal averages. To quantitate the effect of eplerenone on GFAP expression, we chose three sections from each DRG randomly, quantified the GFAP intensity in cellular areas, and then subtracted the background intensity to get relative intensity.

**Acute Cell Dissociation and Whole Cell Patch Recording in Small-diameter Cells**

**Cell Culture.** Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). The bilateral L4 and L5 DRGs were isolated, and the sheath was carefully removed in ice-cold normal artificial cerebrospinal fluid (composition below). The connective tissue was digested by exposure to Ca2+-free bath solution containing 1.0% collagenase type IA (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C followed by three washes in normal bath solution. DRGs were then dissociated by trituration with fire-polished Pasteur pipettes. Dissected cells were plated onto poly-D-lysine coated glass coverslips in Neurobasal Medium (Life Technologies, Grand Island, NY) containing 1% glutamine, 2% B-27 supplement, and 1% penicillin-streptomycin. DRG cells were incubated at 37°C for at least 4 h before recording.

**Electrophysiological Recording.** After 4–12 h culture, coverslips were transferred to a recording chamber and DRG cells were visualized under differential interference contrast using an inverted microscope (IX71; Olympus America Inc., Center Valley, PA). Whole cell current clamp recordings of small DRG neurons were conducted at room temperature with an AxoPatch-200B amplifier (Molecular Devices Corp., Sunnyvale, CA). Patch pipettes (2.5–4.0 mΩ) were fabricated from borosilicate glass. The recording chamber was continuously perfused at room temperature with oxygenated bath solution at a flow rate of 2 ml/min. Data were acquired on a Pentium IV computer with Clampex 9.0 program (Molecular Devices). After Ω2-seal formation, the whole cell configuration was obtained at room temperature under voltage clamp with a holding potential of −60 mV, then switched to current clamp mode. Data were low-pass filtered at 10 kHz and obtained only from small-sized neurons (≤ 30 μm diameter), which are primarily nociceptors. Cells were considered healthy and included if they exhibited resting membrane potentials more negative than −40 mV and action potential (AP) overshoot above 20 mV.

Excitability measurements included the threshold current (rheobase), AP threshold, resting membrane potential (Vm), AP maximum rising rate (V/s), amplitude and duration of
after hyperpolarization, and input resistance. Vm was measured 1 min after a stable recording was obtained. Current pulses from −0.2 to 0.37 nA (80-ms pulse duration) were delivered in increments of 0.3 nA until one or more APs were evoked. Longer suprathreshold pulses (1 s) were then applied to determine whether cells could fire multiple APs. The threshold current (rheobase) was defined as the minimum current required to evoke an AP. The AP voltage threshold was defined as the first point on the upstroke of an AP at which dV/dt was 10% of the maximum. AP duration was measured at threshold.

**Solutions and Chemical Application.** The artificial cerebrospinal fluid used for dissection contained (in mM) NaCl 130, KCl 3.5, CaCl₂ 1.2, MgCl₂ 1.2, NaH₂PO₄ 1.25, CaCl₂ 1.2, MgCl₂ 1.2, NaHCO₃ 24, and glucose 10, pH adjusted to 7.3 with NaOH, bubbled with 95% O₂/5% CO₂ before use. The normal bath solution for recording excitability parameters contained (in mM) NaCl 140, CaCl₂ 2, MgCl₂ 1, HEPES 10, and glucose 25. The pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mM) KCl 140, CaCl₂ 1, MgCl₂ 2, NaOH 10, ethylene glycol tetraacetic acid 11, Mg-adenosine triphosphate 2, Li-guanosine-5′-triphosphate 1, HEPES 10; pH was adjusted to 7.3 with KOH, and the osmolarity was adjusted to approximately 90% of the bath solution. Voltages were corrected for the liquid junction potential, which was estimated to be 5.6 mV. For experiments in cultured cells, eplerenone was made up from a 10 mM dimethyl sulfoxide stock solution, and aldosterone (Sigma-Aldrich) was made up from a 10 mM 95% ethanol stock solution. Treated cells were compared with vehicle-treated controls.

**Statistical Analysis**
Statistical analyses were performed using Graphpad Prism software (Graphpad Prism, La Jolla, CA) and Sigmastat software (Systat Software, Chicago, IL). Comparison of values between different experimental groups was done using nonparametric methods for data that did not show a normal distribution based on the D’Agostino and Pearson omnibus normality test. Comparison of multiple groups was done using ANOVA (parametric) or Kruskal-Wallis test (nonparametric). Comparison of two groups was done using Student t-test (parametric) or Mann-Whitney (nonparametric). Time course data were evaluated with repeated measure ANOVA. The statistical test used is indicated in the text or figure and table legends. Significance was ascribed for P value less than 0.05. Levels of significance are indicated by the number of symbols, e.g., *, P = 0.01 – <0.05; **, P = 0.001 – 0.01; ***, P value less than 0.001 (Figs. 2, 4, 5, 6, 7, and Tables 1 and 2). Data are presented as average ± SEM.

**Results**

**The MR Is Expressed in the DRG Neurons and Translocates into the Nucleus Early after Inflammation**
Both MR and GR were detected in complimentary DNA from whole DRG from quantitative polymerase chain reaction experiments, but their expression was not significantly regulated after local inflammation on postoperative day (POD) 3 at the message RNA level (P = 0.61). Immunohistochemistry of DRG slices showed that the MR immunoreactivity was expressed in all neurons. In normal DRG, MR immunoreactivity was mainly observed in the cytoplasm (figs. 1 and 2). As this localization was somewhat unexpected (see Discussion), the predominantly cytoplasmic location of the MR in normal DRG sections was confirmed using a different antibody, a monoclonal antibody (ID5) directed against the A/B region in the N-terminal (n = 4 animals).

After inflammation of the L4/5 DRG, MR translocation to the nucleus of neurons in the L4/5 DRG was observed. This translocation was maximal on POD 1, gradually decreasing back to normal on POD 14 (figs. 1 and 2). This time course suggested that nuclear MR might be particularly important in initiating pain behaviors. The nuclear
translocation on POD 1 was unlikely to be entirely due to some systemic effect of the DRG inflammation: in DRG taken on POD 1 from the T12 level (n = 4 animals), remote from the inflamed L4/L5 DRG, only 19 ± 4% of cells showed nuclear localization, compared with 63 ± 13% in the inflamed L5 DRG. The nuclear localization in T12 DRG was not significantly different from that observed in normal noninflamed L4/L5 DRG. We also found that MR immunoreactivity also sharply increased in the membrane on POD 7 in the inflamed DRG, and was still increased on POD 14 (figs. 1 and 2).

**Eplerenone Decreased Satellite Glia Activation in the Inflamed DRG**

Satellite glia activation in the sensory ganglia often occurs under pathological conditions i.e., after peripheral nerve injury or local DRG inflammation.8,19,20 When compared with the normal DRG, we found that glia activation, as measured by increased immunoreactivity for GFAP, was significantly increased throughout inflamed DRG (POD 1) without eplerenone. However, the inflamed DRG with local eplerenone treatment showed a significantly decreased satellite glial activation (figs. 3 and 4).

**In Vivo: Local Eplerenone Reduced Pain Behaviors Induced by DRG Inflammation**

To study the effects of MR blockade in vivo, in one group of animals eplerenone was included in the zymosan/IFA used to inflame the DRG. A second group received zymosan/IFA with cholesterol as a chemically similar control without hormonal activity. A third group received the same amount of eplerenone placed subcutaneously under the back, instead of locally in the DRG, to control for possible systemic effects of eplerenone. All three groups showed decreased withdrawal threshold with von Frey filament testing starting from POD 1 (fig. 5A). In the cholesterol and systemic eplerenone groups, the large rapid increase in mechanical sensitivity was similar to that previously observed with zymosan/IFA staining results from experiments as shown in figure 3. N = 3–4 animals per group; individual values for each animal are shown along with the mean and SEM. Each individual animal value is the average of 20–30 observations. * Relative intensity of zymosan-inflamed dorsal root ganglia (on postoperative day 1, Zym) significantly different from normal dorsal root ganglia and from zymosan-inflamed dorsal root ganglia treated with local eplerenone (Zym + Epl). (ANOVA with Holm-Sidak posttest.)
inflammation without cholesterol. The group with local eplerenone in the DRG had significantly less mechanical inflammation without cholesterol. The group with local eplerenone treatment significantly reduced the pain behaviors induced by dorsal root ganglion inflammation. (A) Mechanical threshold (von Frey testing): animals locally treated with Eplerenone added to the zymosan/incomplete Freund adjuvant (IFA) used to inflame the LS dorsal root ganglion (Zym + Epl) had a significantly smaller reduction in ipsilateral threshold compared with animals inflamed with zymosan/IFA + cholesterol (inactive control) (“Zym + Cho”) or with animals in which the Eplerenone was applied systemically with zymosan/IFA + cholesterol (inactive control) (“Zym + Cho”). N = 7–13 animals per group (includes animals from the von Frey testing group).

**Fig. 5.** Local eplerenone treatment significantly reduced the pain behaviors induced by dorsal root ganglion inflammation. (A) Mechanical threshold (von Frey testing): animals locally treated with Eplerenone added to the zymosan/incomplete Freund adjuvant (IFA) used to inflame the LS dorsal root ganglion (Zym + Epl) had a significantly smaller reduction in ipsilateral threshold compared with animals inflamed with zymosan/IFA + cholesterol (inactive control) (“Zym + Cho”) or with animals in which the Eplerenone was applied systemically subcutaneously under the skin of the back (Zym + Epl [s. c.]). N = 7–13 animals per group. * Significantly different from Zym + Cho group on that day; # significantly different from systematic Eplerenone given group on that day (two-way repeated measures (RM) ANOVA with Bonferroni posttest). (B) Rearing behavior (number of rears/5 min) induced by introduction into a novel chamber at time 0 in the same experimental groups plus an unoperated group (naive), tested on postoperative day 1. * Significantly different from naive group at that time point (two-way RM ANOVA with Bonferroni posttest). In this figure, the number of symbols indicates the level of significance (see Methods). N = 7–13 animals per group (includes animals from the von Frey testing group).

As a more complex, nonreflex behavioral measure, we previously demonstrated that LID caused a reduction in the rearing normally observed during the first few minutes after placement of the rat in a novel environment. This effect of LID was reversed by the antiinflammatory drug naproxen, suggesting it is pain related. In this study, a similar reduction in rearing was observed in the Zym + Cho group tested on POD 1. This was largely reversed by local but not systemic eplerenone (fig. 5B).

**In Vitro: Inflammation-induced Excitability Effects on Small DRG Neurons Were Partially Reversed by Eplerenone**

Membrane properties were recorded in acutely isolated (after 8–12 h in primary culture) small-diameter DRG neurons using the current clamp configuration of the whole cell patch clamp (fig 6; table 1). Cells isolated from normal DRG (“control”) were compared with cells isolated on POD 1 after inflammation (“Zym”). We observed the following.

1. Increased excitability of “Zym” cells compared with control cells; rheobase decreased, the number of APs evoked by suprathreshold current injections increased, and the resting potential was more depolarized.
2. These excitability parameters of cells isolated on POD 1 were normalized by treating with eplerenone (10 μM) in vitro during the 8–12 h culture period; mean rheobase and resting potential were restored to normal level, and the number of APs showed a trend (P = 0.1) toward normalization. After eplerenone treatment, the electrophysiological parameters measured were not significantly different from those in normal cells.
3. No significant effects on electrophysiological parameters were observed in cells from normal DRG treated with eplerenone (10 μM) in vitro during the 8- to 12-h culture period.

Treatment of neurons (N = 14) from inflamed DRG with a lower dose of eplerenone, 1 μM, generally gave intermediate values (between those observed with 0 and 10 μM) for the variables shown in figure 6; however, the differences between the two eplerenone doses were not statistically significant with the exception of the effect on Vm (P = 0.02, t test).

**In Vitro: Aldosterone Induced the Increased Excitability of Small DRG Neurons**

The above results could be interpreted most simply by assuming that some of the excitatory effects of LID were due to activation of the MR, which in normal DRG is inactive. This would explain why eplerenone could reverse most excitatory effects in neurons isolated from LID animals, but had no effect in normal animals. By this interpretation, activation of the MR in cells from normal DRG would be expected to have excitatory effects. To test this, we applied aldosterone, the natural high-affinity agonist of MR, in vitro (fig 7; table 2). Acutely isolated small-diameter DRG neurons were recorded under the current clamp configuration of the whole cell patch clamp. Cells isolated from normal DRG neurons were incubated with or without aldosterone at five different doses (0, 1, 10, 100, and 1,000 nM) for 4–8 h before recording. We observed that isolated neurons incubated with aldosterone showed a U-shaped dose-dependent response in the number of APs evoked by
suprathreshold current injections. The evoked response to current injection peaked at 10 nM (P value less than 0.05) with smaller responses at 1, 100, and 1,000 nM.

**Cultured Neurons Also Show More Nuclear MR When Isolated from Inflamed DRG**

Immunostaining of coverslips confirmed that MR could be detected in neurons. The nuclear staining was more marked in cells isolated from inflamed DRG (POD 1) (fig. 8). Increased nuclear staining could also be observed after treating cells cultured from normal DRG with the MR agonist aldosterone.

**Discussion**

Inflammatory processes are important participants in the pathophysiology of low back pain. Previous studies
implicated the MR in mediating the inflammation observed in vessels, heart, and renal cortex of rodent models of diabetes and hypertension. There is increasing evidence to suggest that MR activation increases the risk and severity of inflammation (for review see reference 5). Nonetheless, to our knowledge, few studies have examined the role of MR activation in pathological pain at the level of the DRG. In this study, we showed that local inflammation of the DRG with zymosan/IFA leads to enhanced pain behaviors starting as early as POD 1, when mechanical hypersensitivity and reduced novelty-induced rearing are also observed. Interestingly, the local inflammation of the DRG is accompanied by the rapid nuclear translocation of MR in DRG neurons on POD 1, which is generally considered to indicate MR activation. Pain behaviors persisted for at least 2 weeks in this study (fig. 5), and were still evident at 8 weeks in our previous study.7 We evaluated the effect of adding eplerenone simultaneously with zymosan/IFA, and found that eplerenone partially reversed these pain behaviors; eplerenone-treated animals returned to the preinflammation baseline after 1 week. Consistent with previous reports, these results suggest that MR activation is involved in the local inflammatory process in the DRG, which triggers pain in this model; hence, MR blockade has an analgesic function.

The mechanisms by which selective MR blockers such as eplerenone are antinociceptive are not completely understood. Eplerenone was only effective when applied locally to the inflamed DRG; the same amount given systemically did not affect pain behaviors, implicating MR receptors in the DRG. Eplerenone may exert its antinflammatory effects indirectly by inhibiting the release of proinflammatory cytokines from neurons, glia, or immune cells. The MR is known to promote M1 inflammation characterized by high levels of oxidative metabolites and proinflammatory cytokines as well as tissue damage; in a previous study, microarray experiments on POD 3 showed upregulation of 6 of 10 selected M1 markers21 in locally inflamed DRG. However, our results also suggest that at least some eplerenone effects may be mediated by direct effects on sensory neurons because eplerenone effects were also observed in cultured neurons, where effects of other cell types should be reduced or diluted. We found marked increases in excitability (decreased rheobase, increased number of APs during a suprathreshold current injection) of small sensory neurons, which could be observed in neurons removed from the inflamed DRG on POD 1 and maintained in primary culture for 8–12 h. These were similar to effects previously reported for cells isolated on POD 3.10 The nuclear translocation of MR in inflamed DRG was also preserved in cells cultured on POD 1. The increased excitability could be partially reversed by treating the cells in vitro with eplerenone. These results suggest that the MR located in neurons may play important roles in establishing pain directly through excitability increases as are seen following DRG inflammation. One possible mechanism is the activation of the proinflammatory nuclear factor-kB transcription factor, which has been shown to mediate proinflammatory effects of MR in some other tissues22–24; nuclear factor-kB has excitatory and pronociceptive effects in DRG neurons (e.g., see references 25,26).

The finding that eplerenone reduced GFAP expression in satellite glia (generally used as a marker of activation) in inflamed DRG might be attributed to indirect general antinflammatory effects, because GFAP upregulation is

| Table 1. Membrane Properties of Isolated Small-diameter Neurons Treated with Eplerenone |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Properties                      | Control         | Control + EPL   | Zym             | Zym + EPL       |
| No. of cells                    | 21              | 17              | 22              | 12              |
| Diameter, μm                    | 25.4 ± 0.3      | 24.2 ± 0.4      | 24.6 ± 0.4      | 24.9 ± 0.6      |
| Cell capacitance, pF            | 25.9 ± 1.3      | 23.4 ± 0.8      | 25.4 ± 1.2      | 23.6 ± 0.9      |
| Resting potential, mV           | −53.9 ± 1.3     | −54.3 ± 0.8     | −47.4 ± 0.9***  | −51.4 ± 1.2     |
| Input resistance, MΩ            | 399 ± 48        | 471 ± 35        | 300 ± 25        | 305 ± 92        |
| Max dV/dt, Mv/μs                | 96.2 ± 6.3      | 108.9 ± 6.6     | 111.3 ± 6.9     | 84.4 ± 9.4†     |
| Max V value, mV                 | 56.3 ± 2.1      | 59.9 ± 1.8      | 64.5 ± 2.4*     | 52.7 ± 3.6†     |
| AP threshold, mV                | −9.7 ± 0.9      | −7.5 ± 0.7      | −7.6 ± 1.1      | −6.1 ± 1.0      |
| AP rheobase, pA                 | 361.9 ± 42.7    | 337.6 ± 20.4    | 202.7 ± 18.6**  | 379.2 ± 35.4††  |
| AP width, mV                    | 3.9 ± 0.2       | 3.3 ± 0.2       | 4.4 ± 0.2       | 3.9 ± 0.3       |
| AHP amplitude, mV               | −61.6 ± 0.9     | −59.8 ± 0.6     | −62.3 ± 1.2     | −59.0 ± 1.2     |
| No. of evoked spikes            | 4.0 ± 1.0       | 6.6 ± 0.8       | 14.2 ± 2.8**    | 7.4 ± 1.8       |

None of the differences between Zym + EPL and control groups were significant. None of the differences between control and control + EPL groups were significant. One-way ANOVA with Bonferroni posttest (diameter, cell capacitance, resting potential) or Kruskal-Wallis test with Dunn posttest (all other variables).

*Significant difference between Zym and control groups (number of symbols indicates significance level). †Significant difference between Zym + EPL and Zym groups (number of symbols indicates significance level).

AHP = afterhyperpolarization; AP = action potential; EPL = eplerenone-treated cells; Zym = cells isolated from zymosan-inflamed dorsal root ganglia on postoperative day 1.
Eplerenone Reduces Effects of DRG Inflammation

Fig. 7. Effects of in vitro aldosterone (Aldo) on excitability of acutely cultured small-diameter neurons. Cells were isolated from normal dorsal root ganglia, and Aldo was added during the culture period (4–8 h before recording) at the indicated concentrations. (A) Examples of multiple action potentials evoked by suprathreshold current pulses. (B) Dose response of isolated neurons to Aldo. * Significantly different from control ANOVA.

observed in inflammatory conditions. Activity-dependent somatic release (especially of adenosine triphosphate) from sensory neurons provides a mechanism for neuron-satellite glia communication that may be enhanced in the inflamed DRG, where excitability and spontaneous activity are increased. Because experiments with activity blockers show that abnormal neuronal activity plays a role in satellite glia activation, the observed eplerenone effects on neurons could also play a role in the reduced GFAP expression. Alternatively, or in addition, eplerenone could also have had a direct effect on the satellite glia. In the central nervous system, glia have been shown to express the MR.

Our immunohistochemistry results indicated that the MR was not primarily located in the nucleus in normal DRG, translocating there only early after DRG inflammation. For the classical nuclear actions of the MR receptor, such translocation is generally taken as evidence for activation. The observations in normal DRG may seem to contradict the general view that the MR in most tissues should be chronically activated by basal plasma levels of corticosterone (except in tissues such as kidney where corticosterone is enzymatically degraded)—the affinity of the MR for corticosterone is higher than its affinity for aldosterone, so the (much higher) basal plasma levels of corticosterone should chronically activate the MR. RNA for the enzyme that degrades corticosterone in classical aldosterone-sensitive tissues, 11-hydroxysteroid dehydrogenase type II, is present in DRG though it is not known if this is neuronal or perhaps associated with vascular cells. In addition, the MR can apparently have forms that are less sensitive to corticosterone. For example, studies of MR in brain as well as other tissues suggest that the MR that is in or closely associated with the plasma membrane has a lower affinity for corticosterone; hence, this form of the receptor is not chronically activated. This form may mediate some of the fast, excitatory nongenomic effects of the MR in neurons. Further studies are needed to understand what endogenous agonist causes the MR translocation to the nucleus after DRG inflammation in our model. One possibility is that systemic corticosteroid levels increase due to the pain model (e.g., as observed in some other pain models), enough to activate the MR. However, if this were the case, translocation should have occurred in all DRG, but we found that DRGs several levels above the inflamed DRG did not show nuclear translocation on POD 1. Alternatively, locally produced endogenous steroids could activate the receptor, or the local inflammation process could somehow modify the MR receptor or associated proteins so that it has sensitivity to basal corticosterone levels more like that observed in other tissues. Finally, it is worth noting that the apparent subcellular localization of the MR can vary depending on what antibody is used, presumably because ligand or interacting proteins may interfere with binding of a particular antibody, or because a given antibody may only recognize certain conformations of the protein. The antibody used in this study was derived from an antigen based on the last (C-terminal) 20 amino acids of the MR sequence, which is in the ligand-binding domain. The unexpected pattern of MR localization we observed in normal DRG may seem to contradict the general view that the MR in most tissues is present in DRG sensitive tissues, 11-hydroxysteroid dehydrogenase type II, is present in DRG though it is not known if this is neuronal or perhaps associated with vascular cells. In addition, the MR can apparently have forms that are less sensitive to corticosterone. For example, studies of MR in brain as well as other tissues suggest that the MR that is in or closely associated with the plasma membrane has a lower affinity for corticosterone; hence, this form of the receptor is not chronically activated. This form may mediate some of the fast, excitatory nongenomic effects of the MR in neurons. Further studies are needed to understand what endogenous agonist causes the MR translocation to the nucleus after DRG inflammation in our model. One possibility is that systemic corticosteroid levels increase due to the pain model (e.g., as observed in some other pain models), enough to activate the MR. However, if this were the case, translocation should have occurred in all DRG, but we found that DRGs several levels above the inflamed DRG did not show nuclear translocation on POD 1. Alternatively, locally produced endogenous steroids could activate the receptor, or the local inflammation process could somehow modify the MR receptor or associated proteins so that it has sensitivity to basal corticosterone levels more like that observed in other tissues. Finally, it is worth noting that the apparent subcellular localization of the MR can vary depending on what antibody is used, presumably because ligand or interacting proteins may interfere with binding of a particular antibody, or because a given antibody may only recognize certain conformations of the protein. The antibody used in this study was derived from an antigen based on the last (C-terminal) 20 amino acids of the MR sequence, which is in the ligand-binding domain. The unexpected pattern of MR localization we observed in normal DRG was somewhat similar to that described in amygdala neurons using an antibody that, like the one we used, recognizes the ligand-binding domain. The above discussion assumes that the MR effects studied in the patch clamp experiments were genomic, nuclear effects. Our studies were designed to examine such effects; aldosterone and eplerenone were applied for at least 4–8 h (pretreatment) but were not present during the recording. In addition, our electrophysiological studies were consistent with these assumptions: no effect of eplerenone was observed in normal cells (which had little nuclear receptor), but aldosterone applied to normal cells had excitatory effects. In hippocampal neurons the rapid nongenomic effects of steroids...
mediated by plasma membrane MR can be rapidly washed out,33 and hence even if present should not have been observed in our recordings. However, in other systems, some of the membrane-MR effects can be prolonged, often tending to reinforce the genomic effects.32 We cannot completely rule out the possibility that long-lasting but nongenomic effects contributed to our findings.

The dose–response curve for aldosterone effects in electrophysiological experiments seemed to be an inverted U-shape, with excitatory effects at 1 and 10 nM. The declining effects at higher doses could be due to activation of the GR. In some tissues including macrophages and microglia6 and some brain regions,31 MR and GR have opposing effects. This may also be true at the level of the spinal cord where neuronal MR is also found. In one study, using intrathecal injection, agonists of GR but antagonists of MR had antinociceptive effects;35 however, the role of spinal GR in pain is controversial (e.g.,34,36).

Inflammation is a component of many different models of both inflammatory and neuropathic pain, not just low back pain models. This study presents evidence that the MR may play important roles in pain acting at the level of peripheral sensory neurons. It will be of interest to determine the possible effects of MR antagonists in other types of pathological pain. The MR is a potentially novel target for pain therapeutics, particularly because drugs targeting MR are already approved by the United States Food and Drug Administration for other clinical uses.

The authors thank Mark Baccei, Ph.D., Assistant Professor, Department of Anesthesiology, and James Herman, Ph.D., Professor, Department of Psychiatry, both at the University of Cincinnati College of Medicine, Cincinnati, Ohio, for helpful discussions. 1D5 antibody was the kind gift of Elise Gomez-Sanchez, Ph.D., Professor, Department of Endocrinology, University of Mississippi Medical Center, Jackson, Mississippi.

### References

1. Badley EM, Rasooly I, Webster GK: Relative importance of musculoskeletal disorders as a cause of chronic health conditions as used for electrophysiological recordings. Neurons isolated from normal dorsal root ganglia showed less MR expression in the nucleus compared with neurons isolated from inflamed dorsal root ganglia (postoperative day 1; Zym) or with neurons isolated from normal dorsal root ganglia and treated in vitro with 10 nM aldosterone (Aldo). Scale bar = 50 μm. Zym = zymosan.


