Dissociation of Dorsal Root Ganglion Neurons Induces Hyperexcitability That Is Maintained by Increased Responsiveness to cAMP and cGMP

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Zheng J-H, Walters ET, Song X-J. Dissociation of dorsal root ganglion neurons induces hyperexcitability that is maintained by increased responsiveness to cAMP and cGMP. J Neurophysiol 97: 15–25, 2007. First published October 18, 2006; doi:10.1152/jn.00559.2006. Injury or inflammation affecting sensory neurons in dorsal root ganglia (DRG) causes hyperexcitability of DRG neurons that can lead to spontaneous firing and neuropathic pain. Recent results indicate that after chronic compression of DRG (CCD treatment), both hyperexcitability of neurons in intact DRG and behaviorally expressed hyperalgesia are maintained by concurrent activity in cAMP-protein kinase A (PKA) and cGMP-protein kinase G (PKG) signaling pathways. We report here that when tested under identical conditions, dissociation produces a pattern of hyperexcitability in small DRG neurons similar to that produced by CCD treatment, manifest as decreased action potential (AP) current threshold, increased AP duration, increased repetitive firing to depolarizing pulses, increased spontaneous firing and resting depolarization. A novel feature of this hyperexcitability is its early expression—as soon as testing can be conducted after dissociation (~2 h). Both forms of injury increase the electrophysiological responsiveness of the neurons to activation of cAMP-PKA and cGMP-PKG pathways as indicated by enhancement of hyperexcitability by agonists of these pathways in dissociated or CCD-treated neurons but not in control neurons. Although inflammatory signals are known to activate cAMP-PKA pathways, dissociation-induced hyperexcitability is unlikely to be triggered by signals released from inflammatory cells recruited to the DRG because of insufficient time for recruitment during the dissociation procedure. Inhibition by specific antagonists indicates that continuing activation of cAMP-PKA and cGMP-PKG pathways is required to maintain hyperexcitability after dissociation. The reduction of hyperexcitability by blockers of adenyl cyclase and soluble guanylyl cyclase after dissociation suggests a continuing release of autocrine and/or paracrine factors from dissociated neurons and/or satellite cells, which activate both cyclases and help to maintain acute, injury-induced hyperexcitability of DRG neurons.

INTRODUCTION

Damage to the peripheral nervous system produces long-lasting hyperexcitability of sensory neurons in diverse species (Walters 1994). In mammals, injury affecting the axons or somata of sensory neurons having their somata in dorsal root ganglia (DRGs) often causes hyperexcitability that may lead to spontaneous firing, neuropathic pain, and paresthesias (Devor 1994; Zimmermann 2001). Although electrophysiological mechanisms contributing to the expression of hyperexcitability in DRG neuronal somata after injury to the peripheral nervous system have been investigated intensively, less is known about the signals that induce and maintain this hyperexcitability (Ji and Strichartz 2004). Recently we showed that two signaling pathways are important for maintaining both DRG neuronal hyperexcitability and behaviorally expressed hyperalgesia in an animal model of neuropathic pain: chronic compression of rat dorsal root ganglia (CCD treatment) (Song et al. 2006). During CCD treatment, somata of DRG neurons are mechanically compressed and probably exposed to inflammatory mediators (Ma et al. 2006). One signaling pathway found to maintain CCD effects was the cAMP-protein kinase A (PKA) pathway (Song et al. 2006), which is known to be involved in the hyperalgesia and hyperexcitability of DRG neurons’ somata and peripheral terminals stimulated by inflammatory signals (e.g., Akins and McCleskey 1993; Aley and Levine 1999; Caterina et al. 1997; Cui and Nicol 1995; England et al. 1996; Evans et al. 1999; Ferreira and Nakamura 1979; Fowler et al. 1985; Gold et al. 1996; Hingtgen et al. 1995; Lopshire and Nicol 1998; Taiwo et al. 1989). The second signaling pathway found to maintain CCD effects was the cGMP-protein kinase G (PKG) pathway (Song et al. 2006). This was interesting because, in acute experiments, the cGMP-PKG pathway has more often been associated with depressive effects on DRG neuronal excitability (e.g., Duarte et al. 1992; Kress et al. 1996; Liu et al. 2004; Sachs et al. 2004) than with sensitizing effects (Aley et al. 1998; Parada et al. 2005; Vivancos et al. 2003; see also Tegeder et al. 2004).

A surprising finding in our investigation of cAMP-PKA and cGMP-PKG contributions to CCD-induced hyperalgesia and DRG neuronal hyperexcitability was that these pathways had little effect on behavior or soma excitability in the absence of CCD treatment (Song et al. 2006). Although this observation appeared to conflict with earlier reports of CCD-induced DRG neuronal hyperexcitability, our excitability tests were performed on sensory neuron somata that remained in place within excised but intact ganglia, whereas previous studies implicating the cAMP-PKA pathway in hyperexcitability had been performed either on dissociated DRG neuronal somata (Akins and McCleskey 1993; Aley et al. 1998; Caterina et al. 1997; Cui and Nicol 1995; England et al. 1996; Evans et al. 1999; Gold et al. 1996; Lopshire and Nicol 1998; Rathee et al. 2002; Smith et al. 2000) or on neurons in excised ganglia that had previously been compressed (Hu et al. 2001). This suggested that injury-related stress, caused in these cases by either dissociation or compression of DRG neurons, induces an increase in electrophysiological responsiveness to both cAMP and cGMP, and this increased responsiveness is important for...
maintaining hyperexcitability. The possibility that dissociation of DRG neurons produces cAMP- and cGMP-dependent hyperexcitability is also important because it would suggest that these sensitizing responses do not require the recruitment of inflammatory cells for their induction or early maintenance and might involve mechanisms intrinsic to injured neurons, as has been demonstrated in an invertebrate nociceptor (Ambron et al. 1996; Bedi et al. 1998) and strongly implicated in dissociated DRG neurons (Ma and LaMotte 2005). Here we show that dissociation of DRG neurons produces acute cAMP- and cGMP-dependent hyperexcitability that is remarkably similar to that produced by chronic compression of the DRG. Some of the results of the present study have been published in abstract form (Song et al. 2005).

Methods

Surgical procedures

All investigations were conducted in conformity with the APS’s “Guiding Principles in the Care and Use of Animals” and were approved by the Parker College Research Institute’s Committee on the Care and Use of Experimental Animals. Experiments were performed on 66 adult, male Sprague-Dawley rats (120–150 g). The rats were housed in groups of four in plastic cages (40 × 60 × 30 cm) with soft bedding and free access to food and water under a 12-h day/12-h night cycle. They were kept 3–7 day under these conditions, before and ±10 day after surgery. All surgeries were done under pentobarbital sodium (50 mg/kg ip) anesthesia. Hollow stainless-steel, L-shaped rods (4 mm in length and 0.6 mm in diameter) were surgically implanted unilaterally into the intervertebral foramen (IVF) at L4 and L5 to chronically compress the DRG (CCD treatment) (see Song et al. 1999, 2003a, 2006). In brief, rats (n = 33) were anesthetized, paraspinal muscles were separated from the mamiillary and transverse processes and the IVF of L4 and L5 was exposed. A rod was implanted chronically into the IVF. After surgery, the muscle and skin layers were sutured. Sham surgery (n = 33) involved surgical procedures identical to those described but without insertion of the rod.

Behavioral testing

Thermal hyperalgesia was indicated by a decrease in the latency of foot withdrawal evoked by a radiant heat stimulus as described previously (Hargreaves et al. 1988; Song et al. 2003, 2006). In brief, each rat was placed in a box containing a smooth glass floor maintained at 26 ± 0.5°C. Radiant heat was focused on part of the hindpaw that was flush against the glass and delivered until the hindpaw moved (or ±20 s to prevent tissue damage). The latency of foot withdrawal in naïve, control rats is 9–12 s (Song et al. 2003a, 2006). Thermal stimuli were delivered four times to each hind paw at 5- to 6-min intervals. Withdrawal latencies were normalized by subtracting each value on the treated side from the corresponding value on the contralateral side and the results were expressed as difference scores. The rats were tested on each of two successive days prior to surgery. Postoperative tests were conducted 1, 3, 7, and 10 day after surgery and/or on the day of electrophysiological recording (day 3–10). Thermal hyperalgesia for a given rat was defined as a postoperative decrease of foot withdrawal latency from the mean preoperative value, and/or on the day of electrophysiological recording (day 3–10). Thermal hyperalgesia was indicated by a decrease in the latency of thermal withdrawal for a given rat was defined as a postoperative decrease of foot withdrawal latency from the mean preoperative value, and/or on the day of electrophysiological recording (day 3–10).

Excised, intact ganglion preparation

Some DRG neurons were tested while still in place in excised ganglia prepared as described previously (Song et al. 2003a, 2006), using L4 and/or L5 ganglia in 18 CCD and 18 sham-operated rats. Briefly, each rat was anesthetized with pentobarbital sodium after its final behavioral test. The sciatic nerve was transected at the mid-thigh level, and its proximal portion traced to the ganglia. A laminectomy was performed, and the location of the rod in CCD rats checked. Ice-cold, oxygenated, buffered solution containing (in mM) 140 NaCl, 3.5 KCl, 1.5 CaCl2, 1 MgCl2, 4.5 HEPES, 5.5 HEPES-Na, and 10 glucose (pH 7.3; osmolality, 310–320 mosM) was dripped onto the surface of the ganglion during the procedure. The ganglia from the left side of the L4 and L5 segments were removed and placed in 35-mm petri dishes containing ice-cold, oxygenated, buffered solution. The perineurium and epineurium were peeled off, and the attached sciatic nerve and dorsal roots were transected adjacent to the ganglion. The otherwise intact ganglion was then treated with collagenase (Boehringer type P, 1 mg/ml) for 30 min at 35°C, transferred to the recording chamber, and mounted on the stage of an upright microscope (BX50-WI, Olympus, Japan). A U-shaped stainless steel rod with four crossing silver wires held the ganglion gently in place. The DRG was incubated in the oxygenated, buffered solution at room temperature (~22°C). Five to 16 cells were tested electrophysiologically in each ganglion.

Dissociation of DRG neurons

DRG neurons were dissociated from L4 and/or L5 ganglia taken from 13 CCD and 15 sham surgery rats using conventional methods (e.g., Vydyanathan et al. 2005; Xu and Huang 2002). In brief, the excised ganglion was minced using microdissection scissors, the DRG fragments transferred into 10 ml of the buffered solution containing collagenase (type IA, 1 mg/ml, Sigma) and trypsin (0.5 mg/ml, Sigma), and then incubated for 30 min at 35°C. The DRG fragments were removed, rinsed five to six times in the buffered solution and put into the buffered solution (5 ml) containing DNase (0.2 mg/ml, Sigma) to prevent possible toxicity from DNA leaking from ruptured cells. Individual neurons were dissociated by passing DRG fragments through a set of fire-polished glass pipettes with decreasing diameter. The dissociated cells were then transferred to acid-cleaned glass coverslips, and electrophysiological tests were performed at 22°C 2–6 h after dissociation.

Whole cell current- and voltage-clamp recordings

Whole cell patch-clamp recordings were made with an Axoclamp 2B amplifier (Sunnyvale, CA) from small DRG neurons (soma diameter: 15–30 μm; membrane input capacitance: ≤45 pF), which largely correspond to neurons with C-fiber conduction velocities (Ma et al. 2003). Conduction velocity was not measured in the present study. Glass electrodes were fabricated with a Flaming/Brown micropipette puller (P-97, Sutter instruments). Electrode impedance was 3–5 MΩ when filled with saline containing (in mM) 120 K+–glucuronate, 20 KC1, 1 CaCl2, 2 MgCl2, 11 ethylene-glycol-bis-(β-aminoethyl-ether) N,N,N',N'-tetraacetic acid (EGTA), 2 Mg-ATP, and 10 HEPES-K (pH 7.2; osmolality, 290–300 mosM). Electrode position was controlled by a three-dimensional (3-D) hydraulic micromanipulator (MHW-3, Narishige). When the electrode tip touched the cell membrane, gentle suction was applied to form a tight seal (serial resistance >2 GΩ). Under ~70 mV command voltage, additional suction was applied to rupture the cell membrane. After obtaining the whole cell mode, the recording was switched to bridge mode (I = 0) and the resting membrane potential (RMP) was recorded.

All neurons accepted for analysis had a RMP of ~−40 mV or more negative. To compare the excitability of the intact and dissociated DRG neurons from CCD or sham control rats, we examined the RMP, action potential (AP) current threshold, afterhyperpolarization (AHP), repetitive discharge evoked by depolarizing current, and other electrophysiological properties of DRG neurons. The RMP was taken 2–3 min after a stable recording was first obtained. AP properties were
measured by delivering intracellular currents from −0.1 to 0.5 nA (50-ms pulses) in increments of 0.02–0.1 nA. AP current threshold was defined as the minimum current required to evoke an AP. AP voltage threshold was defined as the first point on the upstroke at which the rising rate exceeded 50 mV/ms. AP amplitude was measured from the voltage threshold to the peak, and AP duration was measured at AP voltage threshold. AHP amplitude was measured from the RMP to the peak hyperpolarization, and AHP duration was measured as the interval from onset until the AHP had recovered by 50%. The whole cell input resistance \( R_{\text{in}} \) was calculated on the basis of the steady-state \( I-V \) relationship during a series of 100-ms hyperpolarizing currents delivered in steps of 0.01–0.02 nA from −0.2 to 0.1 nA. Input capacitance \( C_{\text{m}} \) was calculated by integration of the capacity transient evoked by a 10-mV pulse in voltage-clamp mode. Repetitive discharge was measured by counting the spikes evoked by 1-s, intracellular pulses of depolarizing current normalized to 2.5 times the AP threshold current. All electrophysiological recordings and data analyses were conducted by experimenters blind to previous pharmacological or compression treatment of the cells (Song et al. 2003a, 2006).

**Drug application**

Contributions of cAMP-PKA and cGMP-PKG pathways to hyperexcitability of dissociated and previously compressed DRG neurons were investigated by applying the following drugs in the bath to excised DRG or to dissociated DRG neurons at final concentrations of 100 \( \mu \text{M} \): a cAMP analogue, 8-Br-cAMP; a PKA activator, Sp-cAMPS; an adenylyl cyclase inhibitor, SQ22536; a PKA inhibitor, Rp-cAMPS; a cGMP analogue, 8-Br-cGMP; a PKG activator, Sp-cGMPs; and a PKG inhibitor, Rp-8-pCPT-cGMPs. The soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), was dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution and subsequently diluted with oxygenated HEPES-artificial cerebrospinal fluid (ACSF) before application. The final concentrations of ODQ and DMSO were 10 \( \mu \text{M} \) and 0.01%. DMSO at 0.01% produces no significant effects on RMP or excitability of either intact DRG neurons (Song et al. 2006) or acutely dissociated DRG neurons (data not shown). Although we have not conducted systematic dose-response studies on these drugs, all were previously found to have electrophysiological effects at doses between 10 and 500 \( \mu \text{M} \) in excised ganglia (Song et al. 2006). Each was diluted in the buffered solution just before application or diluted initially with distilled water for a stock solution that was subsequently diluted with oxygenated buffered solution prior to application. Application of all drugs began 10–30 min prior to and continued during the 3–4 h of electrophysiological recording. In most experiments, only one drug was applied to each DRG; but in one study, the DRG neurons were pretreated with an inhibitor of either the cAMP-PKA or cGMP-PKG pathway and then an activator of the same pathway was given 20 min later.

**Statistical tests**

Comparisons among sham-intact, CCD-intact, CCD-dissociated, and sham-dissociated groups were performed with one-way ANOVA followed by Newman-Keuls tests. Specific hypotheses about differences between each drug-treated group and its corresponding control group were tested with individual \( t \)-test for each electrophysiological parameter examined. Fisher’s exact test was used to identify differences in the incidence of spontaneous activity between each stressed or drug-treated group and its corresponding control group. All data are presented as means ± SE. Statistical results are considered significant if \( P < 0.05 \).

**RESULTS**

**Dissociation and CCD treatment produce similar hyperexcitability of DRG neurons**

To make direct comparisons to previous studies linking the cAMP-PKA pathway to hyperexcitability of DRG neurons (see Discussion) and to confirm that dissociation produces hyperexcitability of DRG neurons, we compared the electrophysiological properties of DRG neurons tested 2–6 h after dissociation to those tested in intact ganglia in concurrent experiments under identical patch recording conditions at 22°C. Whole cell current-clamp recordings were performed on 22 and 28 small DRG neurons in intact ganglia from three sham-treated and four CCD-treated rats, respectively, and on 27 and 30 small dissociated DRG neurons from four sham-operated and four CCD-treated rats, respectively. All CCD-treated rats included in these studies demonstrated thermal hyperalgesia, expressed as a latency for foot withdrawal on the side of DRG compression that was ≥3 s shorter than the latency on the contralateral side (see Methods). Electrophysiological properties of these four groups are compared in Table 1. Dissociated neurons taken from both the sham-operated and CCD-treated rats exhibited increased input resistance \( R_{\text{in}} \) and decreased input capacitance \( C_{\text{m}} \) compared with neurons tested in intact DRG, as would be expected from the decrease in cell volume caused by disconnection of the axon from the soma during dissociation. Interestingly, for many of the electrophysiological properties (Fig. 1 and Table 1), neurons tested in intact DRG from sham-operated animals (our minimally stressed controls) were significantly different from neurons in the other three groups that had experienced injury-related stress; i.e., dissociated neurons from sham-operated animals, neurons in intact DRG from CCD-treated animals, and dissociated neurons from CCD-treated animals. This was true for RMP, AP current threshold, AP duration, repetitive firing, and the incidence of spontaneously active neurons. In each case, pairwise comparisons (after 1-way ANOVA) showed that the sham-dissociated, CCD-intact, and CCD-dissociated groups were not significantly different from each other but were significantly different from the sham-intact group. Four properties did not fit this pattern: AP voltage thresholds, AP amplitudes, and AHP amplitudes were not significantly different among any of the groups, and AHP duration was significantly enhanced by dissociation and by dissociation after CCD treatment (\( P < 0.01 \) in each case) but not by CCD treatment without dissociation. Previously we found (Song et al. 2006) that CCD effects on AP voltage thresholds in intact DRG depended on RMP, being much more prominent when cells with RMP more depolarized than −50 mV were excluded. Because both dissociation and CCD treatment often depolarize RMP to values more positive than −50 mV, we used a cutoff of −40 mV in the present study.

The consistent pattern of effects on AP current threshold, AP duration, repetitive firing, spontaneous activity, and RMP demonstrate that acute dissociation and CCD treatment produce qualitatively and quantitatively similar short-term changes in excitability and RMP and that when these stresses are combined, there is little additivity in their electrophysiological effects.

Similar changes in RMP, AP current threshold, repetitive discharge, and spontaneous activity have been observed after
TABLE 1. Effects of dissociation and CCD treatment on membrane properties of small DRG neurons

<table>
<thead>
<tr>
<th></th>
<th>Sham Intact</th>
<th>Sham Dissociated</th>
<th>CCD Intact</th>
<th>CCD Dissociated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>22</td>
<td>27</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>20.1 ± 1.6</td>
<td>20.9 ± 1.5</td>
<td>22.6 ± 1.2</td>
<td>24.0 ± 0.8</td>
</tr>
<tr>
<td>R_in, MΩ</td>
<td>416 ± 14</td>
<td>635 ± 12**</td>
<td>425 ± 15</td>
<td>651 ± 14**</td>
</tr>
<tr>
<td>C_in, pF</td>
<td>43.2 ± 1.1</td>
<td>35.5 ± 0.9**</td>
<td>41.2 ± 1.0</td>
<td>36.4 ± 0.7***</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>−55.9 ± 1.5</td>
<td>−49.8 ± 0.9**</td>
<td>−50.3 ± 1.0*</td>
<td>−49.2 ± 1.1**</td>
</tr>
<tr>
<td>AP current threshold (nA)</td>
<td>0.34 ± 0.04</td>
<td>0.12 ± 0.02**</td>
<td>0.07 ± 0.01**</td>
<td>0.08 ± 0.01**</td>
</tr>
<tr>
<td>AP voltage threshold, mV</td>
<td>−27.2 ± 1.7</td>
<td>−27.7 ± 1.0</td>
<td>−27.9 ± 1.1</td>
<td>−29.6 ± 1.0</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>62.1 ± 1.0</td>
<td>59.6 ± 1.0</td>
<td>60.0 ± 0.8</td>
<td>58.8 ± 0.8</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>3.43 ± 0.28</td>
<td>4.65 ± 0.27*</td>
<td>4.58 ± 0.31*</td>
<td>4.65 ± 0.29*</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>21.4 ± 3.5</td>
<td>24.9 ± 1.9</td>
<td>20.5 ± 3.1</td>
<td>27.6 ± 3.2</td>
</tr>
<tr>
<td>AHP duration, ms</td>
<td>13.6 ± 3.5</td>
<td>32.6 ± 5.2**</td>
<td>14.4 ± 2.6</td>
<td>36.2 ± 7.0**</td>
</tr>
<tr>
<td>Number of APs, 1-s pulse</td>
<td>2.05 ± 0.25</td>
<td>8.11 ± 1.57**</td>
<td>7.14 ± 1.19**</td>
<td>8.67 ± 1.34**</td>
</tr>
<tr>
<td>Neurons exhibiting SA#</td>
<td>0.0%</td>
<td>12.9%*</td>
<td>12.5%*</td>
<td>14.3%*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Asterisks indicate significant differences from the sham intact group; *, P < 0.05, **, P < 0.01 (1-way ANOVA followed by pairwise comparisons using Newman-Keuls tests; comparisons of incidence (%) are by Fisher’s exact test). n, number of dorsal root ganglion (DRG) neurons tested; R_in, input resistance; C_in, input capacitance; RMP, resting membrane potential; AP, action potential; AHP, afterhyperpolarization; SA, spontaneous activity; CCD, chronic compression of the DRG. Number of APs monitors repetitive firing to a standardized 1-s depolarizing pulse, #, SA neurons were not included in n, and were not included in any of the other data sets above.

other forms of injury to DRG neurons (e.g., Abdulla and Smith 2001a; Gallego et al. 1987; Gurtu and Smith 1988; Song et al. 2003a; Stebbing et al. 1999; Study and Kral 1996). In the following studies, these four electrophysiological properties were used to test our general hypothesis that cAMP-PKA and cGMP-PKG pathways contribute to hyperexcitability of DRG neurons produced by dissociation and were used to compare these contributions to those associated with hyperexcitability after CCD treatment.

Dissociation and CCD treatment enhance electrophysiological responsiveness of DRG neurons to agonists of the cAMP-PKA and cGMP-PKG pathways

The first half of our general hypothesis about cAMP-PKA and cGMP-PKG contributions to injury-related hyperexcitability of DRG neurons proposes that injury increases the electrophysiological responsiveness of DRG neurons to activation of these pathways (Song et al. 2006). To test this proposition, we compared effects of membrane-permeant agonists of each pathway on small DRG neurons in the four groups described in the preceding text: minimally stressed neurons in intact DRG from sham-implanted animals, dissociated neurons from sham-operated animals, neurons in intact DRG from CCD-treated animals, and dissociated neurons from CCD-treated animals.

Figure 2 illustrates these comparisons graphically across four electrophysiological properties and shows the similarity in pattern of effects produced by each agonist on each of the three stressed groups. Agonists of the cAMP-PKA pathway, 8-Br-cAMP and Sp-cAMPS, and of the cGMP-PKG pathway, 8-Br-cGMP and Sp-cGMP (each 100 µM), produced no significant effects on excitability in the minimally stressed, sham-intact group, replicating at 22°C with the whole-cell patch technique our previous observations made at 36°C with sharp electrodes (Song et al. 2006). In all groups except the sham-intact group, each of these drugs significantly depolarized RMP relative to neurons within the same group tested without drugs (P < 0.05 in each case) and relative to RMP in the sham-intact group tested without drugs (P < 0.01 in each case). Similarities among the three stressed groups were also observed with the other electrophysiological properties measured. Agonists of the cAMP-PKA and cGMP-PKG pathways lowered AP current threshold relative to neurons within the same group tested without drugs (P < 0.05 in each case) and relative to threshold...
in the sham-intact group tested without drugs ($P < 0.01$ in each case). Similarly, all of these agonists increased repetitive firing evoked by a standardized test pulse relative to neurons within the same group tested without drugs ($P < 0.05$ in each case), and relative to repetitive firing in the sham-intact group tested without drugs ($P < 0.01$ in each case). In many cases (Fig. 2D), the agonists increased spontaneous activity relative to that displayed by neurons within the same group tested without drugs ($P < 0.05$), and the agonists always increased spontaneous activity relative to that seen in the sham-intact group tested without drugs ($P < 0.01$). Taken together, these results show that dissociation and CCD treatment enhance electrophysiological responsiveness of the cells to both the cAMP-PKA and cGMP-PKG signaling pathways and to a very similar degree.

To verify that the enhancement of hyperexcitability produced by these agonists results from specific actions on the cAMP-PKA and cGMP-PKG pathways, we tested to see if specific antagonists of these pathways would block the effects of the corresponding agonists. Pretreatment of dissociated DRG neurons from sham-treated rats with Rp-cAMPS or Rp-8-pCPT-cGMPS (each 100 μM) prevented the increase in excitability produced by the corresponding agonists, 8-Br-cAMP or 8-Br-cGMP (each 100 μM) delivered 20 min later. No significant differences were found between neurons treated with Rp-cAMPS alone ($n = 27$) and neurons treated with Rp-cAMPS followed by 8-Br-cAMP ($n = 13$) in RMP ($-55.5 \pm 1.3$ vs. $-54.4 \pm 1.7$ mV), AP threshold current ($0.21 \pm 0.03$ vs. $0.19 \pm 0.04$ nA), repetitive firing during a 1-s depolarizing pulse ($2.8 \pm 0.6$ vs. $2.5 \pm 0.7$ spikes), and incidence of SA (0 vs. 0%). Similarly, no significant differences were found between neurons treated with Rp-8-pCPT-cGMPS alone ($n = 21$) and neurons treated with Rp-8-pCPT-cGMPS followed by 8-Br-cGMP ($n = 15$) in RMP ($-57 \pm 1.1$ vs. $-56.2 \pm 1.6$ mV), AP threshold current ($0.24 \pm 0.04$ vs. $0.23 \pm 0.03$ nA), repetitive firing ($1.7 \pm 0.3$ vs. $2.1 \pm 0.3$ spikes), and incidence of SA (0 vs. 0%). The data for Rp-cAMPS alone and Rp-8-pCPT-cGMPS alone are also shown in Fig. 3 (labeled “S-D RpA” and “S-D RpG”, respectively). The blockade of the effects of these agonists by the corresponding antagonists strengthen our conclusion that dissociation and CCD treatment enhance the electrophysiological responsiveness of DRG neurons to both the cAMP-PKA and cGMP-PKG signaling pathways.

**FIG. 2.** Effects of agonists of the cAMP-protein kinase A (PKA) pathway (*left*) and cGMP-protein kinase G (PKG) pathway (*right*) on RMP and excitability in DRG neuron somata after sham treatment (S-I, tested in intact DRG), dissociation (S-D), CCD treatment (C-I, tested in intact DRG), or CCD treatment followed by dissociation (C-D). Also shown are comparisons of these treatments in the absence of agonists. Applied agonists of the cAMP-PKA pathway were 8-Br-cAMP (8BrcA, 100 μM) and Sp-cAMPS (SpcA, 100 μM). Agonists of the cGMP-PKG pathway were 8-Br-cGMP (8BrcG, 100 μM) and Sp-cGMPS (SpcG, 100 μM). A: effects on RMP. B: effects on the current needed to reach AP threshold. C: effects on repetitive firing evoked by a 1-s depolarizing pulse at 2.5 times the threshold current. D: percentage of tested neurons displaying spontaneous AP activity. *, $P < 0.05$; **, $P < 0.01$ indicate significant differences compared with the sham-intact (S-I) control group. #, $P < 0.05$; ##, $P < 0.01$ indicate significant differences between corresponding groups in the presence and absence of the indicated agonist. The numbers of cells tested in each group are shown in parentheses.
Hyperexcitability and spontaneous activity in DRG somata after either dissociation or CCD treatment require ongoing activation of cAMP-PKA and cGMP-PKG pathways

The second half of our general hypothesis about contributions of cAMP-PKA and cGMP-PKG pathways to injury-related hyperexcitability of DRG neurons proposes that continuing activation of these pathways is important for maintaining injury-related hyperexcitability (Song et al. 2006). We tested this proposition by applying membrane-permeant antagonists of each pathway and seeing if excitability and spontaneous firing would be reduced in neurons in any of the four groups described above. Figure 3 summarizes the results and shows the similarity in pattern of effects produced by each antagonist on properties of the three stressed groups: intact, CCD-treated; dissociated; and dissociated + CCD-treated. Antagonists of the cAMP-PKA pathway were SQ22536 (SQ, 100 μM) and Rp-cAMPS (RpA, 100 μM). Antagonists of the cGMP-PKG pathway were 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM) and Rp-8-pCPT-cGMPS (RpG, 100 μM). A: effects on RMP. B: effects on the current needed to reach AP threshold. C: effects on repetitive firing evoked by a 1-s depolarizing pulse at 2.5 times the threshold current. D: percentage of tested neurons displaying spontaneous activity. *, P < 0.05; **, P < 0.01 indicate significant differences compared with the sham-intact (S-I) control group. #, P < 0.05; ##, P < 0.01 indicate significant differences between corresponding groups in the presence and absence of the indicated agonist.

FIG. 3. Comparisons of effects of antagonists of the cAMP-PKA pathway (left) and cGMP-PKG pathway (right) on RMP and excitability in DRG neuronal somata after sham treatment (S-I, tested in intact DRG), dissociation (S-D), CCD treatment (C-I, tested in intact DRG), or CCD treatment followed by dissociation (C-D). For comparison, the data summarizing responses in the absence of drugs in Fig. 2 are repeated here. Applied antagonists of the cAMP-PKA pathway were SQ22536 (SQ, 100 μM) and Rp-cAMPS (RpA, 100 μM). Antagonists of the cGMP-PKG pathway were 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM) and Rp-8-pCPT-cGMPS (RpG, 100 μM).
repetitive firing also showed tendencies (albeit not statistically significant) to be incomplete. The lack of complete inhibition might reflect a submaximal dose of antagonist but is also consistent with additional pathways contributing to the injury-related hyperexcitability. Nonetheless, the large reduction of the electrophysiological effects of dissociation and CCD treatment by antagonists of the cAMP-PKA and cGMP-PKG pathways provides strong evidence for an important contribution of these two pathways to the maintenance of hyperexcitability and spontaneous firing shortly after both forms of injury-related stress.

**DISCUSSION**

*Dissociation and CCD treatment produce similar hyperexcitability of DRG neurons*

Because neuronal dissociation necessarily involves close axotomy, it would not be surprising for it to trigger some of the same neuronal responses that are evoked by other forms of injury. This was first reported in nociceptive sensory neurons of *Aplysia* (Ambron et al. 1996; Bedi et al. 1998; Liao et al. 1999; Sung et al. 2004) where dissociation produces long-lasting decreases in soma AP threshold and increases in both repetitive firing and AP duration that are not accounted for by the decrease in cell volume and that are similar to changes in the same neurons produced by peripheral axotomy in the intact animal (Gasull et al. 2005; Ungless et al. 2002; Walters et al. 1991). Ma and LaMotte (2005; cf. Zhang et al. 1999) showed, using sharp electrodes at 36°C, that dissociation-induced hyperexcitability also occurs in small DRG neurons, which correspond to sensory neurons that are often nociceptive and have C-fiber conduction velocities (Ma et al. 2003; Zhang et al. 1999). We have used whole cell patch recording methods at 22°C to show that both acute dissociation of DRG neurons and prolonged CCD treatment not only decrease AP threshold, depolarize the neurons, and increase $R_{\text{in}}$, as observed by Ma and LaMotte (2005) and Ma et al. (2006), but also increase AP duration, increase repetitive firing to prolonged depolarizing pulses, and increase the incidence of spontaneous firing (Fig. 1). Most of these effects (decreased AP threshold, increased repetitive firing and spontaneous activity, slight depolarization) increase excitability. The AP broadening, if it also occurs in synaptic terminals, may enhance transmitter release. The larger number and greater magnitude of effects of dissociation found in the present study than in those by Ma and LaMotte (2005), Ma et al. (2006), and Tan et al. (2006) might be explained by differences in time of testing (2–6 vs. 3–8 and 24–30 h after dissociation), RMP cut-off for inclusion of neurons (−40 vs. −45 mV), temperature (22 vs. 36°C), recording method (patch vs. sharp electrodes), and/or culture conditions (uncoated glass vs. polyornithine- and laminin-coated glass). Interestingly, the lack of additivity we found between the acute dissociation effects and CCD effects suggests that each set of effects may have been expressed at near maximal levels under our conditions. Hyperexcitability and AP broadening in DRG neuronal somata are also produced by peripheral nerve injury or inflammation (Abdulla and Smith 2001a, b; Djouhri et al. 2001; Kim et al. 1998; Liu et al. 2000; Ma et al. 2003; Stebbing et al. 1999; Wall and Devor 1983; Xu et al. 2000), raising the question of how closely related these various hyperexcitability states are. Interactions or occlusion between dissociation-induced hyperexcitability and hyperexcitability produced by other manipulations may result in experimental underestimates of some forms of hyperexcitability examined in dissociated neurons, which might confound mechanistic analyses (e.g., see Flake et al. 2004 for unexpected mechanistic questions encountered in acutely dissociated DRG neurons).

Our demonstration that the hyperexcitable effects of dissociation and CCD treatment are expressed at 22°C will be useful for investigations of the underlying biophysical mechanisms, which may be quite complex (see Table 1). Patch-clamp studies of DRG neuron conductances are usually performed at room temperature rather than body temperature because the slower kinetics facilitate the experimental separation of different currents. Future studies of the ionic mechanisms underlying dissociation- and CCD-induced hyperexcitability will benefit from direct comparisons to numerous patch-clamp studies that have been performed at room temperature. Some of these have demonstrated alterations of specific DRG neuron conductances that are strong candidates for contributions to dissociation- and CCD-induced hyperexcitability. For example, Tan et al. (2006) have described altered Na$^+$ and K$^+$ conductances in medium-sized DRG neurons after CCD treatment, and complex alterations of Na$^+$ and K$^+$ conductances have also been found in small DRG neurons after peripheral axotomy (e.g., Abdulla and Smith 2001b, 2002; Flake et al. 2004; Yang et al. 2004). A potentially important difference between the hyperexcitability of DRG neurons observed after other injury-related manipulations and that observed after dissociation in the present study is the rapid onset of expression after dissociation (≈2 h). This brief latency suggests that transcriptional alterations are not necessary for acute hyperexcitability caused by injury.

*Dissociation enhances electrophysiological responsiveness to agonists of the cAMP-PKA and cGMP-PKG pathways*

We found that dissociated DRG neurons respond to agonists of both the cAMP-PKA and cGMP-PKG pathways with a further increase in excitability, expressed as a dramatic decrease in AP threshold, a large increase in repetitive firing, and an increase in spontaneous activity. In addition, each agonist significantly depolarized RMP (an effect not observed in intact DRG under different recording conditions) (see Song et al. 2006). The added hyperexcitability caused by the agonists was indistinguishable from that produced by the same agonists in concurrent experiments conducted with whole cell patch recording at 22°C on neurons in intact DRG after CCD treatment (Fig. 2) and was similar to what we observed earlier with sharp electrodes at 36°C in intact DRG after CCD treatment (Song et al. 2006). In both studies, agonists of these pathways had only weak, statistically insignificant effects on the excitability of minimally stressed somata in intact DRG that had not received CCD treatment. It is unlikely that these differences reflect increased access of the agonists to the DRG neurons after dissociation or CCD treatment because the soma membranes of all the tested neurons, including neurons recorded on the surface of minimally stressed, intact DRG, were directly exposed to the bath after preparation for whole cell patch recording by enzyme treatment and surgical removal of the perineurium and epineurium.
These results indicate that dissimilar forms of injury-related stress—acute dissociation and prolonged compression of the DRG—greatly enhance the electrophysiological responsiveness of the stressed neurons to activity in the cAMP-PKA and cGMP-PKG pathways. An interesting possibility is that the enhancement of excitatory effects of inflammatory mediators on DRG neurons by prior nerve injury (Song et al. 2003b) or CCD treatment (Ma et al. 2006) involves an increased responsiveness within the neuron to the cAMP-PKA pathway because excitatory effects of some inflammatory mediators are mediated by this pathway (e.g., Aley and Levine 1999; Ferreira and Nakamura 1979; Taiwo et al. 1989). The cAMP-PKA pathway may also act synergistically with other signaling pathways because a combination of inflammatory signals (prostaglandin E₂, serotonin, bradykinin, and histamine, which should activate multiple signaling pathways, including the cAMP-PKA pathway) produces excitatory effects on neuronal somata in intact DRG from uninjured animals (Ma et al. 2006). It should be emphasized that in the absence of injury, the responsiveness to cAMP-PKA activity in other regions of the sensory neurons (notably the peripheral terminals) might be greater than we found in the somata (see Aley and Levine 1999; Ferreira and Nakamura 1979; Taiwo et al. 1989). One implication of our findings in dissociated neurons is that previous demonstrations of cAMP-PKA contributions to hyperexcitability of DRG somata (e.g., Aley et al. 1998; Cardenas et al. 2001; England et al. 1996; Evans et al. 1999; Gold et al. 1996; Ingram and Williams 1996; Lopshire and Nicol 1998; Rathee et al. 2002; Smith et al. 2000) may have depended at least partly on dissociation increasing the electrophysiological responsiveness of the DRG neurons to cAMP.

The close similarity between the hyperexcitable effects produced by the cAMP-PKA and cGMP-PKG pathways after injury is interesting, given the opposing effects that can be produced by these pathways in DRG neurons (e.g., Dontchev and Letourneau 2003; McGeehe et al. 1992). Where these pathways converge to modulate DRG neuron excitability and how they interact are important questions that need further investigation. Nonetheless, the potent blockade of the effects of the cAMP-PKA and cGMP-PKG agonists by pretreatment with the corresponding antagonists and the similar degrees of enhancement produced by the same concentrations of each of the different agonists (as well as the similar degrees of inhibition produced by different antagonists) suggest that the effects we have attributed to either the cAMP-PKA pathway or cGMP-PKG pathway are not explained by pharmacological cross-activation of the other pathway. The increased electrophysiological responsiveness to the cAMP-PKA and cGMP-PKG pathways after dissociation or DRG compression may be part of a larger pattern of altered cellular signaling following injury and inflammation. For example, an acute inflammatory stimulus to peripheral terminals of DRG neurons “primes” hyperalgesic responses to a separate, subsequently applied inflammatory stimulus by inducing a novel linkage between cAMP and a specific PKC isoform (Parada et al. 2005). Such findings suggest that complex interactions among the numerous cellular signals that induce and maintain hyperexcitability of DRG neurons may be complicated further by injury- or inflammation-dependent plasticity within some of the signaling pathways.
2003; Sun et al. 2006; White et al. 2005; Zhang et al. 1995). Factors released from neuronal cells may have autocrine actions on the releasing neurons, which might be particularly important after dissociation. Nonneuronal cells were also present after dissociation in our studies (often associated with large neurons), and, although we washed the cultures extensively before testing, they were not superperfused during testing, so the possibility exists that paracrine factors released during testing affected the dissociated neuron responses. However, the concentration of any released paracrine factors would have been quite low because we did not test dissociated DRG neurons that were in physical contact with other cells, and the density of all cells in vitro was much lower than in situ. Because DRG dissociation was performed rapidly, before inflammatory cells could be recruited, mediators released by infiltrating inflammatory cells were unlikely to contribute significantly to dissociation-induced hyperexcitability of DRG neurons. The possibility remains, however, that mediators released from a small number of inflammatory cells resident in the DRG contributed.

The cGMP synthesis maintaining DRG neuron hyperexcitability might be in the DRG neurons (e.g., Bauer et al. 1993; Sung et al. 2006; Wood et al. 1989) or in satellite glia (e.g., Morris et al. 1992; Shi et al. 1998), where cGMP would have to promote the release of paracrine factors that then produce hyperexcitability of DRG neurons. Both nitric oxide synthase (NOS) (Morris et al. 1992; Shi et al. 1998) and PKG (Qian et al. 1996; Sung et al. 2006) occur in DRG neurons. Again, the much lower density of cells after acute dissociation and the similar magnitude of cGMP-PKG effects in situ and in vitro suggest that NO activating the cGMP-PKG pathway in DRG neurons after injury may do so as an autocrine factor. Although NO synthesized in DRG neurons has been suggested to act primarily in a paracrine fashion on satellite glia to promote neuronal survival (reviewed by Thippeswamy and Morris 2001a), neuronal sites of cGMP synthesis that may contribute to hyperexcitability have escaped detection (see also Shi et al. 1998). For example, small amounts of cGMP might produce large effects if neuronal sGC and PKG are located in close proximity to their cellular targets. Defining the sites of action of these enzymes, and the interactions of the cGMP-PKG pathway with the cAMP-PKA pathway and other signaling pathways after injury will be important for understanding fundamental hyperexcitability mechanisms that contribute to neuropathic pain. It will be interesting to see if these pathways also induce longer-lasting alterations of DRG neuron excitability that depend on altered protein synthesis and gene transcription, as found in nociceptive sensory neurons in Aplysia (Lewin and Walters 1999; Sung et al. 2004).

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