Ionic Mechanisms Underlying Inflammatory Mediator-Induced Sensitization of Dural Afferents

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Migraineurs experience debilitating headaches that result from neurogenic inflammation of the dura and subsequent sensitization of dural afferents. Given the importance of inflammatory mediator (IM)-induced dural afferent sensitization to this pain syndrome, the present study was designed to identify ionic mechanisms underlying this process. Trigeminal ganglion neurons from adult female Sprague Dawley rats were acutely dissociated 10–14 d after application of retrograde tracer DiI onto the dura. Modulation of ion channels and changes in excitability were measured in the absence and presence of IMs (in μM : 1 prostaglandin, 10 bradykinin, and 1 histamine) using whole-cell and perforated-patch recordings. Fura-2 was used to assess changes in intracellular Ca^{2+} . IMs modulated a number of currents in dural afferents, including those both expected and/or previously described [i.e., an increase in tetrodotoxin-resistant voltage-gated Na^+ current (TTX-R I_{Na}) and a decrease in voltage-gated Ca^{2+} current] as well currents never before described in sensory neurons (i.e., a decrease in a Ca^{2+} -dependent K^+ current and an increase in a Cl^- current), and produced a sustained elevation in intracellular Ca^{2+} . Although several of these currents, in particular TTX-R I_{Na} , appear to contribute to the sensitization of dural afferents, the Cl^- current is the primary mechanism underlying this process. Activation of this current plays a dominant role in the sensitization of dural afferents because of the combination of the density and biophysical properties of TTX-R I_{Na} , and the high level of intracellular Cl^- in these neurons. These results suggest novel targets for the development of antimigraine agents.

Introduction

Migraine is a neurological disorder characterized by incapacitating head pain. Compelling evidence indicates that neurogenic inflammation of the dura and subsequent dural afferent sensitization are fundamentally important for initiating migraine pain (Ray and Wolff, 1940; Strassman et al., 1996; Sarchielli et al., 2000; Burstein, 2001). However, the ionic mechanisms involved in dural afferent sensitization have yet to be discovered.

A feature that distinguishes nociceptive afferents from all other primary sensory neurons is that they can be sensitized by mediators released at sites of inflammation. Identification of the underlying mechanisms of sensitization remains an active area of investigation because of the critical role this increase in afferent excitability plays in ongoing pain and hypersensitivity (hyperalgesia) observed in the presence of tissue injury. Given the direct link between ion channel activity and neuronal excitability, ion channels have remained a primary focus of this line of investigation. Recent evidence suggests that the specific ion channels underlying the sensitization of nociceptive afferents varies as a function of target of innervation. For example, there are differ-

ences between the mechanisms underlying the acute sensitization of afferents innervating the colon and those innervating glabrous skin (Gold and Traub, 2004). Similar differences have been described for afferents innervating the muscle (Harriott et al., 2006), ileum (Stewart et al., 2003), bladder (Yoshimura and de Groat, 1999), and colon (Beyak et al., 2004) in response to persistent inflammation. Although the differences described to date suggest the involvement of distinct neurobiological processes, increases in voltage-gated Na^+ and/or decreases in voltage-gated or Ca^{2+} -dependent K^+ currents appear to be mechanisms common to sensitization of nociceptive afferents in these previous studies. In marked contrast to the results from these previous studies, we recently described inflammatory mediator (IM)-induced changes in dural afferents that appeared to reflect processes in addition to those previously described (Harriott and Gold, 2009). Given that IM-induced sensitization of this population of afferents appears to play an essential role in the headache associated with migraine (Ray and Wolff, 1940; Strassman et al., 1996; Sarchielli et al., 2000; Burstein, 2001), the purpose of the present study was to identify the mechanisms underlying the IM-induced sensitization of these afferents.

Acutely dissociated retrogradely labeled dural afferents from adult female rats were studied with whole-cell patch-clamp and Ca^{2+} -imaging techniques. Results from this analysis suggest that, in addition to changes in Na^+ currents common to the sensitization of other afferent populations, and the inhibition of a K^+ current that appears to be unique to dural afferents, the primary mechanism of IM-induced sensitization of dural afferents appears to be the activation of a Cl^- current.

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Materials and Methods

Animals

Adult female Sprague Dawley rats (Harlan) weighing between 180 and 290 g were used for all experiments. Rats were housed two per cage at the University of Pittsburgh animal facility on a 12 h light/dark schedule with food and water available *ad libitum*. Before all procedures, animals were deeply anesthetized with an intraperitoneal injection (1 ml/kg) of mixture containing ketamine (55 mg/kg), xylazine (5.5 mg/kg), and acepromazine (1.1 mg/kg). Experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All efforts were used to minimize the total number of animals used.

Afferents innervating the dura were identified as previously described after application of DiI to the dura (Harriott and Gold, 2008). Ten to 14 d after DiI application, trigeminal ganglia were removed, enzymatically treated, and mechanically dissociated as previously described (Harriott et al., 2006). Changes in currents, intracellular Ca^{2+} , and excitability were measured 2–8 h after cells were plated.

Electrophysiology

All whole-cell and perforated-patch-clamp recordings were performed with a HEKA EPC10 amplifier (HEKA). Data were low-pass filtered at 5–10 kHz with a four-pole Bessel filter and digitally sampled at 25–100 kHz. Ionic solutions were chosen to study specific currents in isolation. For all solutions, pH was adjusted to between 7.2 and 7.4 with Tris base (unless otherwise stated) and the osmolality adjusted to between 310 and 325 mOsm with sucrose. Thick-walled borosilicate glass (1.5 mm internal diameter; WPI) electrodes were pulled (Sutter P2000) such that, when filled with electrode solution, the resistance was $<5 \text{ M}\Omega$.

Voltage clamp. A standard protocol was used to facilitate comparisons between neurons. After establishing whole-cell access, membrane resistance and capacitance were determined with hyperpolarizing voltage steps from -60 mV . Baseline data were collected over 2–10 min to ensure the particular current under study was stable as well as to facilitate detection of IM-induced changes. IMs were then applied and changes in currents were monitored. Voltage-clamp protocols were used to assess the impact of IMs on current activation, inactivation and deactivation as well as Ca^{2+} dependence with the specific details of each protocol described in conjunction with the description of the specific currents. To facilitate assessment of the reversal potential for IM-induced currents, a voltage ramp from $+50$ to -100 mV over 100 ms was used. Finally, gramicidin perforated-patch recording was used to assess the resting concentration of intracellular Cl^- in dural afferents. Bath and electrode solutions were constructed to either reflect physiological solutions or to facilitate the study of specific currents in isolation. The details of each solution used are as follows.

To isolate Na^+ currents, the electrode solution was composed of the following (in mM): 100 Cs-methanesulfonate, 40 tetraethylammonium (TEA)-Cl, 5 NaCl, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, 11 EGTA, 2 Mg-ATP, and 1 Li-GTP. Bath solution contained the following (in mM): 35 Na-methanesulfonate, 65 choline-Cl, 30 TEA-Cl, 2.5 CaCl_2 , 5 MgCl_2 , 0.05 CdCl_2 , 10 HEPES, and 10 glucose.

To isolate K^+ currents, the electrode solution was composed of the following (in mM): 110 K-methanesulfonate, 30 KCl, 5 NaCl, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, 11 EGTA, 2 Mg-ATP, and 1 Li-GTP. Bath solution contained the following (in mM): 3 KCl, 130 choline-Cl, 2.5 CaCl_2 , 0.6 MgCl_2 , 0.1 niflumic acid (NFA), 10 HEPES, 10 glucose.

To isolate Ca^{2+} currents, the electrode solution was composed of the following (in mM): 100 Cs-methanesulfonate, 5 Na-methanesulfonate, 40 TEA-Cl, 1 CaCl_2 , 2 MgCl_2 , 11 EGTA, 10 HEPES. Bath solution contained the following (in mM): 100 choline-Cl, 30 TEA-Cl, 2.5 CaCl_2 , 0.6 MgCl_2 , 0.1 NFA, 10 HEPES, 10 glucose.

Initial characterization of IM-evoked currents were performed with an electrode solution composed of the following (in mM): 110 K-methanesulfonate, 30 KCl, 5 NaCl, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, 11 EGTA, 2 Mg-ATP, 1 Li-GTP; and bath solution containing the following (in mM): 3 KCl, 130 NaCl, 2.5 CaCl_2 , 0.6 MgCl_2 , 10 HEPES, 10 glucose.

The contribution of monovalent and divalent cations to the IM-induced current was assessed by manipulating concentrations of K^+ and Na^+ and Ca^{2+} . The contribution of monovalent cations to the IM-induced current was minimized with an electrode solution containing the following (in mM): 100 Cs-methanesulfonate, 30 CsCl, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, 11 EGTA, 2 Mg-ATP, 1 Li-GTP; and bath solution containing the following (in mM): 130 choline-Cl, 2.5 CaCl_2 , 0.6 MgCl_2 , 10 HEPES, 10 glucose.

To further analyze the source of Ca^{2+} responsible for the IM-induced activation of Cl^- currents, four different manipulations were formed as follows: (1) the addition of Cd^{2+} ($50 \mu\text{M}$) to the bath solution to block influx via voltage-dependent Ca^{2+} channels, (2) the addition of ruthenium red to the bath solution to block influx via ligand-gated ion channels, (3) the substitution of BAPTA (10 mM) for EGTA (11 mM) in the electrode solution, and (4) the use of the combination of an electrode solution with Ca^{2+} artificially buffered to $\sim 620 \text{ nM}$ with an electrode solution containing EGTA (1.2 mM), Ca^{2+} (1 mM), and Mg^{2+} (2 mM), in which influx via voltage-gated Ca^{2+} channels (VGCCs) was also blocked by the addition of Cd^{2+} ($50 \mu\text{M}$) to the bath solution. MaxChelator was used to generate estimates of resting free intracellular Ca^{2+} .

The reversal potential of IM-induced currents was determined using gramicidin perforated-patch recordings and a bath solution containing the following (in mM): 100 choline-Cl, 30 TEA-Cl, 2.5 CaCl_2 , 0.6 MgCl_2 , 10 HEPES, 10 glucose.

Current clamp. Excitability was assessed in the presence and absence of test compounds as previously described (Harriott and Gold, 2009). A neuron was considered sensitized if application of a test solution resulted in a hyperpolarization of action potential (AP) threshold, decrease in rheobase, and/or an increase in the response to suprathreshold stimulation $>2 \text{ SDs}$ from the baseline mean. Current injection was used in the present study as a means to bypass natural processes underlying the transduction of what would be primarily mechanical (changes in vessel diameter) and chemical stimuli in the dura. Given evidence that IMs can sensitize transduction processes (i.e., TRPV1) as well as the ion channels underlying action potential initiation and propagation, changes in excitability described in the present study would serve to amplify IM-induced modulation of transduction processes that should also occur at afferent terminals *in vivo*.

Ca^{2+} imaging

Neurons were incubated with $2.5 \mu\text{M}$ Ca^{2+} indicator fura-2 AM ester with 0.025% pluronic as described previously (Lu et al., 2006), and IM-induced Ca^{2+} transients were acquired on a PC running Metafluor software (Molecular Devices) via a CCD camera (Roper Scientific; model RTE/CCD 1300). The ratio (R) of fluorescence emission (510 nm) in response to 340/380 nm excitation [controlled by a lambda 10–2 filter changer (Sutter Instrument)] was acquired at 1 Hz during IM applications.

Drugs

All salts and test compounds were obtained from Sigma-Aldrich. IMs consisted of bradykinin ($10 \mu\text{M}$), histamine ($1 \mu\text{M}$), and prostaglandin E_2 ($1 \mu\text{M}$), where bradykinin was dissolved in 1% acetic acid (23.58 mM stock concentration), prostaglandin E_2 (PGE_2) was dissolved in 100% ETOH (10 mM stock concentration), and histamine was dissolved in water (100 mM stock concentration). All stock solutions were stored at -20°C until the day of use. IM-vehicle bath containing the final concentration of ETOH (0.01%) and acetic acid (0.001%) was used as a control. NFA was dissolved in 100% ETOH. Ruthenium red was dissolved in water.

There were four primary reasons for the use of an “inflammatory soup” in the present study rather than single mediators. First, we (Gold and Traub, 2004; Harriott and Gold, 2009) and, more importantly, others (Strassman et al., 1996; Oshinsky, 2006; Jakubowski et al., 2007; Levy et al., 2008; Edelmayer et al., 2009) have used a mixture to sensitize dural afferents, with the studies performed by others on the afferent terminals. Second, although an important and interesting question, the focus of the study was not on which mediators and/or mediator receptors underlie the sensitization of dural afferents, but which ion channels are downstream from these mediators. Third, data from microdialysis studies of

Figure 1. Inflammatory mediators increase TTX-R Na^+ currents in dural afferents. Na^+ currents were recorded in dural afferents ($n = 13$) before and after IM application. **A**, Two types of Na^+ currents were detected in dural afferents. A high-threshold slowly activating and slowly inactivating TTX-R Na^+ current and a low-threshold rapidly activating and inactivating TTX-S Na^+ current. **B**, Example of the voltage dependence of inactivation and activation when data were fitted with a Boltzmann equation. **C**, After IM application (Post), there was a significant increase in maximal TTX-R Na^+ current density in dural afferents relative to the baseline current density (Pre). Calibration: 2 nA, 2 ms, $*p < 0.05$. **D**, However, there was no significant difference in TTX-S Na^+ current density ($p > 0.05$). Error bars indicate SE.

injured tissue (Hargreaves et al., 1994; Roszkowski et al., 1997; Lepinski et al., 2000) as well as the CSF from migraine patients during a migraine (Sarchielli et al., 2000) indicate that multiple mediators are released at the same time. Furthermore, evidence suggests that a combination of mediators such as mast cell (Levy et al., 2007) and cyclooxygenase (Jakubowski et al., 2005) products contribute to the sensitization of dural afferents and/or migraine. Fourth, there is already evidence that the IM-induced modulation of at least one ion channel (i.e., $\text{Na}_v 1.9$) requires a combination of mediators (Maingret et al., 2008). Therefore, we chose to pool mediators rather than attempt to identify the mediator or specific combination of mediators that underlies the sensitization of dural afferents.

Data analysis

Data were analyzed with PulseFit (HEKA), SigmaPlot, and SigmaStat software (Systat Software). Conductance–voltage (G – V) curves were constructed from I – V curves by dividing the evoked current by the driving force on the current, such that $G = I/(V_m - V_{rev})$, where V_m is the potential at which current was evoked and V_{rev} is the reversal potential for the current that was measured directly (for K^+ and Na^+ currents). Instantaneous I – V data, obtained from the tail currents measured after activation of voltage-gated Ca^{2+} currents, was used to construct G – V curves for voltage-gated Ca^{2+} currents. Activation and steady-state availability data were fitted with a Boltzmann equation of the following form: $G = G_{max}/1 + \exp[(V_{0.5} - V_m)/k]$, where G is observed conductance, G_{max} is the calculated maximal conductance, $V_{0.5}$ is the potential for half-activation or inactivation, V_m is command potential, and k is the slope factor. K^+ currents were corrected for series resistance voltage error. For Ca^{2+} imaging, the IM-induced change in the fluorescence ratio was determined by subtracting the baseline ratio from the peak value. The decay of the IM-induced Ca^{2+} transient was analyzed as time to 50% decay of the peak (T_{50}).

For comparisons of parametric data collected before and after IM application, either a paired t test or repeated-measures ANOVA were used. Otherwise, a Wilcoxon or Friedman test was used for nonparametric analysis. For unpaired comparisons of the percentage reduction in rheobase, a t test was used for parametric data and a Mann–Whitney U for nonparametric analysis. Data were considered statistically significant when $p < 0.05$. All data are represented as mean $\pm$ SE.

Results

Data were collected from 186 dural afferents acutely dissociated from 36 female Sprague Dawley rats. Of these, 131 were studied in voltage clamp and 45 were studied in current clamp and 25 were studied with fura-2-based microfluorimetry. The size distribution of these neurons was similar to that of our previous study (Harriott and Gold, 2009) with a median cell body capacitance of 29.54 pF (with 21.3 and 37.0 as 25th and 75th percentiles).

Inflammatory mediators increase voltage-gated Na^+ currents

We recently described significant increases in the AP overshoot and rate of rise in dural afferents after application of IMs (bradykinin, 10 μM ; histamine, 1 μM ; and prostaglandin E_2 , 1 μM) (Harriott and Gold, 2009). Since VGSCs (voltage-gated Na^+ channels) are primarily responsible for the upstroke of the action potential, we predicted that these IM-induced changes in the AP waveform reflected an increase in Na^+ currents (I_{Na}). For steady-state availability, I_{Na} was elicited with a 15 ms test pulse to -10 mV after a series of 500 ms prepulses from -120 to -5 mV. To isolate tetrodotoxin-resistant currents (TTX-R I_{Na}) from TTX-sensitive currents (TTX-S I_{Na}), a 500 ms prepulse to a potential between -40 and -30 mV was used to inactivate TTX-S I_{Na} . The prepulse potential used for each neuron was based on results from the steady-state availability data for total Na^+ current evoked in each neuron. To validate this approach for separation of TTX-S from TTX-R I_{Na} , TTX (100 nM) was used in five dural afferents and the currents isolated with both methods were identical (data not shown). TTX-S currents were isolated by digitally subtracting TTX-R currents from the total I_{Na} . To examine changes in the voltage dependence of activation, I_{Na} was elicited with test pulses from -60 to $+65$ mV after a 100 ms prepulse to -110 mV. TTX-R I_{Na} was again isolated with a prepulse to a potential between -40 and -30 mV, and TTX-S currents were isolated by digital subtraction (Fig. 1A,B).

As described in other afferent populations, two general types of I_{Na} were detected in dural afferents. These included a relatively low threshold, rapidly activating, rapidly inactivating TTX-S I_{Na} (Fig. 1A) and a relatively high threshold, more slowly activating and inactivating TTX-R I_{Na} (Fig. 1A). No persistent current was detected, although the voltage protocols used were not optimized to detect the presence of a low threshold persistent current. In dural afferents, peak TTX-S I_{Na} density was 28.6 ± 10.6 pA/pF, whereas that of TTX-R I_{Na} was 65.3 ± 19.3 pA/pF at a test potential of -10 mV ($n = 13$). TTX-S and TTX-R I_{Na} were detectable in every dural afferent studied. Parameters describing the voltage dependence of activation and inactivation of TTX-S and -R I_{Na} are summarized in Table 1.

Consistent with our prediction, TTX-R I_{Na} was significantly (i.e., >2 SDs from baseline) increased in 12 of 13 dural afferents after IM application (Fig. 1C). There was no significant increase in TTX-S I_{Na} after IM application (Fig. 1D). There were small but significant IM-induced changes in the biophysical properties of

Table 1. IM-induced changes in the biophysical properties of TTX-R and TTX-S Na^+ currents

	Inactivation				Activation			
	$V_{1/2}$		Slope		$V_{1/2}$		Slope	
	Pre-IM	Post-IM	Pre-IM	Post-IM	Pre-IM	Post-IM	Pre-IM	Post-IM
TTX-R	-26.83 ± 1.4	-27.59 ± 1.9	4.54 ± 0.2	4.34 ± 0.2	-8.46 ± 0.8	$-10.61 \pm 1.0^*$	4.28 ± 0.4	4.08 ± 0.3
TTX-S	-66.59 ± 7.8	-68.3 ± 3.4	9.05 ± 1.0	8.49 ± 2.4	-19.46 ± 3.1	$-21.92 \pm 3.0^*$	7.18 ± 1.1	6.48 ± 1.2

There were small but significant differences in the voltage dependence of activation of TTX-R and TTX-S Na^+ currents after IM application. Both currents displayed a small hyperpolarizing shift in the $V_{1/2}$ of activation after IM.

*Significant difference between pre-IM and post-IM, where $p < 0.05$.

Figure 2. Inflammatory mediators decrease a Ca^{2+} -dependent K^+ current in dural afferents. K^+ currents were elicited from dural afferents ($n = 14$) before and after IM application. In the presence of $50 \mu\text{M}$ Cd^{2+} , to block voltage-gated Ca^{2+} currents, IMs did not reduce K^+ current density at any potential (**A**). Furthermore, IMs did not significantly decrease the maximal conductance in the presence of Cd^{2+} (**B**) and produced no shift in the voltage dependence of activation (**C**). **D**, In the absence of Cd^{2+} , IMs reduced K^+ current density at positive potentials. IMs also significantly decreased the maximal conductance (**E**). However, there was no shift in the voltage dependence of activation of K^+ currents (**F**). I - V and G - V data were corrected for voltage errors associated with uncompensated series resistance. **A**, Inset, Example of K^+ currents elicited in dural afferents with the voltage protocol shown beneath current traces. * $p < 0.05$. Error bars indicate SE.

TTX-R and TTX-S I_{Na} , which included a hyperpolarizing shift in the voltage dependence of activation (Table 1).

Inflammatory mediators decrease a Ca^{2+} -dependent K^+ current

There is a growing body of evidence from a number of investigators that persistent inflammation results in a significant decrease in voltage-dependent K^+ currents (Yoshimura and de Groat, 1999; Stewart et al., 2003; Harriott et al., 2006) and, more relevantly, that inflammatory mediators including PGE_2 (Nicol et al., 1997) and NGF (Zhang et al., 2002) produce a rapid decrease in K^+ current. Therefore, we examined the possibility that decreases in K^+ currents (I_{K}) also contribute to IM-induced dural afferent sensitization. K^+ currents were elicited with 10 mV, 500 ms voltage steps between -60 and $+60$ mV after a 500 ms prepulse to -120 mV. The reversal potential for K^+ was determined by eliciting tail currents with voltage steps from -110 to -50 mV in 10 mV increments, after a test pulse to $+40$ mV. Furthermore, since both voltage- and Ca^{2+} -dependent K^+ currents contribute to primary afferent excitability, currents were recorded in the absence and presence of the voltage-dependent Ca^{2+} channel blocker Cd^{2+} ($50 \mu\text{M}$). IMs had no detectable

influence on voltage-dependent K^+ currents evoked in the presence of Cd^{2+} ($n = 7$), as assessed by the voltage dependence of channel activation (Fig. 2B) and the maximal conductance (Fig. 2B). In contrast, when Cd^{2+} was omitted from the bath solution ($n = 7$), an IM-induced suppression of K^+ current was detected. The result was a significant decrease in maximal conductance for total outward current (Fig. 2C). To begin to identify the channel sensitive to the actions of IMs, the experiment was repeated in the presence of ibuprofen (IbTx) (100 nM ; $n = 3$) to selectively block large-conductance Ca^{2+} -dependent K^+ currents (BK). Pre-application of IbTx failed to occlude the actions of IMs, suggesting BK channels do not underlie the IM-sensitive current. Together, results from this series of experiments suggest that IMs suppress a Ca^{2+} -dependent K^+ current in dural afferents different from that previously described in other sensory neurons.

Inflammatory mediators activate Cl^- currents in dural afferents

Although an increase in I_{Na} and decrease in I_{K} could account for the IM-induced sensitization of dural afferents, given minimal shifts in the voltage dependence of activation of either current and the fact that these channels are not active close to the resting membrane potential, they are unlikely to account for the IM-induced changes in passive electrophysiological properties, in particular, membrane depolarization and decreased input resistance reported recently (Harriott and Gold, 2009). Therefore, to identify the ion channel(s) involved in IM-induced changes in passive electrophysiological properties of dural afferents, IM-activated currents were recorded at a holding potential of -60 mV. Consistent with the observed IM-induced membrane depolarization, a significant increase in inward current (mean, $10.7 \pm 2.9 \text{ pA/pF}$) (Fig. 3A,C) was observed after application of IMs to dural afferents ($n = 7$). To begin to identify the basis for this IM-induced current, current was evoked from a series of holding potentials ranging between -60 and $+10$ mV. Data from this series of experiments ($n = 7$) indicated that the IM-induced current had a reversal potential of approximately -30 mV (Fig. 3B), which was close to the calculated equilibrium potential for Cl^- under our recording conditions (-34 mV). Therefore, we tested the prediction that the IM-induced current was mediated by anion flux. IM-induced currents were measured again after reducing extracellular Cl^- concentration from 140 to

36 mM, which produced an equimolar concentration of Cl^- inside and outside the cell. Consistent with our prediction, the decrease in extracellular Cl^- concentration ($n = 6$) resulted in an IM-induced current with a reversal potential at 0 mV (Fig. 3B) and, consequently, an increase in current density evoked at -60 mV (Fig. 3C). Moreover, bath application of non-selective Cl^- channel blocker NFA ($10 \mu\text{M}$) reversed the IM-induced increase in holding current (Fig. 3C) ($n = 5$). Given the nature of this IM-induced current, we subsequently refer to it as $I_{\text{IM-Cl}}$.

Activation of $I_{\text{IM-Cl}}$ is dependent on an increase in the concentration of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_{\text{in}}$)

Although a Ca^{2+} -dependent Cl^- current has yet to be described in trigeminal ganglion neurons, evidence from nodose ganglion neurons suggests that bradykinin is capable of activating a Cl^- current that is Ca^{2+} dependent (Oh and Weinreich, 2004). Therefore, to assess the contribution of Ca^{2+} to $I_{\text{IM-Cl}}$, currents were recorded using intracellular BAPTA (10 mM) to replace EGTA (11 mM). Consistent with the suggestion that an increase in $[\text{Ca}^{2+}]_{\text{in}}$ is important for activation of the $I_{\text{IM-Cl}}$, the increase in Cl^- current was significantly attenuated by 10 mM BAPTA (Fig. 4A) ($n = 7$).

To determine whether influx through VGCCs provides the source of Ca^{2+} that activates the $I_{\text{IM-Cl}}$, currents were measured under conditions in which Na^+ and K^+ currents were minimized by replacing intracellular K^+ with Cs^+ and extracellular Na^+ with choline, leaving Cl^- and Ca^{2+} unchanged. Currents were elicited with 100 ms test pulses from -70 to $+50 \text{ mV}$ after a 40 ms prepulse to 0 mV to evoke Ca^{2+} influx (Fig. 4B). Driving an increase in intracellular Ca^{2+} with this protocol alone was insufficient to activate a Cl^- current in dural afferents. However, the current-voltage relationship for $I_{\text{IM-Cl}}$ [resolved by digital subtraction (Fig. 4C) ($n = 8$) of currents evoked before and after application of IMs] was clearly altered by this protocol, exhibiting pronounced inward rectification that appeared to reflect an increase in outward current between -30 and 0 mV . Furthermore, $I_{\text{IM-Cl}}$ decreased at voltage steps $>0 \text{ mV}$ (Fig. 4C). These data were consistent with the possibility that Ca^{2+} influx via voltage-gated Ca^{2+} currents facilitates $I_{\text{IM-Cl}}$, particularly at potentials between -30 and 0 mV .

To confirm that the shape of the I - V curve in Figure 4C reflected the influence of Ca^{2+} influx through VGCCs, $I_{\text{IM-Cl}}$ was examined again under recording conditions in which intracellular Ca^{2+} was artificially elevated to $\sim 620 \text{ nM}$ (with the reduction of EGTA to 1.2 mM) and Ca^{2+} influx via VGCCs was blocked by the addition $50 \mu\text{M}$ Cd^{2+} to the bath solution. Despite the elevated intracellular Ca^{2+} concentration in dural afferents studied under these recording conditions ($n = 6$), the NFA-sensitive current at -60 mV was comparable with that observed under our standard whole-cell recording conditions when intracellular Ca^{2+} was buffered to $\sim 45 \text{ nM}$ (i.e., $<1 \text{ pA/pF}$). Furthermore, the IM-induced inward current was comparable with that observed when Ca^{2+} influx was facilitated with a prepulse to 0 mV (Fig. 4C) and in the absence of a prepulse to facilitate Ca^{2+} influx (Fig. 3C). However, under conditions in which Ca^{2+} influx was blocked by Cd^{2+} , the I - V relationship for $I_{\text{IM-Cl}}$ was linear (i.e., no increase in outward current between -30 and 0 mV , and no

Figure 3. Inflammatory mediators increase Cl^- currents in dural afferents. **A**, IM application produced an inward current at -60 mV . **B**, When current was evoked from a series of holding potentials ranging between -60 and $+10 \text{ mV}$ ($n = 7$), the IM-induced current reversed direction at approximately -30 mV ($140 \text{ mM Cl}_{\text{out}}$; filled circles). After lowering the concentration of Cl_{out} (36 mM ; open circles; $n = 6$) to produce an equimolar concentration of Cl^- in and out, the reversal potential for the IM-induced holding current reversed at 0 mV . **C**, There was also a significant increase in the holding current recorded at -60 mV when the driving force for Cl^- was increased by lowering Cl_{out} from 140 to 36 mM . The increase in holding current was reversed by application of $100 \mu\text{M}$ NFA ($n = 5$). *Significant difference between post-IM with 140 and $36 \text{ mM Cl}_{\text{out}}$ and between $140 \text{ mM Cl}_{\text{out}}$ and NFA, where $p < 0.05$. Error bars indicate SE.

decrease in outward current at potentials $>0 \text{ mV}$). These observations further suggest that Ca^{2+} influx via VGCCs may facilitate $I_{\text{IM-Cl}}$ efflux but is neither necessary nor sufficient for the activation of $I_{\text{IM-Cl}}$.

To further substantiate this suggestion and rule out the possibility that Ca^{2+} influx via VGCCs contributes to the activation of Cl^- current secondary to an IM-induced shift in the voltage dependence of activation of VGCCs, we recorded Ca^{2+} currents directly ($n = 5$), before and after application of IMs. High-threshold voltage-gated Ca^{2+} currents were the only currents detected in dural afferents. Although these currents did exhibit rundown, it was monitored until currents were stabilized ($\sim 10 \text{ min}$) before the application of IMs. No evidence of a low-threshold current was detected after application of IMs (Fig. 5B). In contrast, however, application of IMs produced a significant ($p < 0.05$) decrease in peak Ca^{2+} current (Fig. 5A,B). The voltage dependence of Ca^{2+} current activation was examined with the instantaneous current-voltage relationship derived from tail currents. IM application did not shift the voltage dependence of activation of Ca^{2+} currents (Fig. 5C), suggesting a voltage-independent mode of current suppression as has been described by others (Dolphin and Scott, 1987).

There is evidence that bradykinin (Vellani et al., 2001) and PGE_2 (Pitchford and Levine, 1991; Schnitzler et al., 2008) can sensitize TRPV1, providing a source of Ca^{2+} for the activation of $I_{\text{IM-Cl}}$. And although an IM-induced activation of such a current should have impacted reversal potential measurements of $I_{\text{IM-Cl}}$, we sought to rule out Ca^{2+} influx via a TRP channel as a source of Ca^{2+} contributing to the activation of $I_{\text{IM-Cl}}$ at resting membrane potential. Toward that end, neurons were studied in the presence of the nonselective Ca^{2+} channel blocker ruthenium red ($10 \mu\text{M}$; $n = 9$). Although we confirmed the ability of ruthenium red to block capsaicin-evoked currents ($n = 4$) (data not shown), ruthenium red failed to block the IM-induced membrane depolarization, increase in holding current and decrease in input resistance (e.g., IM-induced decrease in input resistance was 74 ± 13 and $52 \pm 22\%$ of baseline in the absence and presence of ruthenium red, respectively; $p > 0.05$). These results suggested an IM-induced release of Ca^{2+} from intracellular stores as a source of Ca^{2+} for the activation of $I_{\text{IM-Cl}}$.

To assess this possibility, we examined the impact of IMs on $[\text{Ca}^{2+}]_{\text{in}}$ in dural afferents measured directly with fura-2. Application of IMs to dural afferents resulted in a dramatic increase in $[\text{Ca}^{2+}]_{\text{in}}$ in 9 of 10 neurons tested (Fig. 6), whereas an IM-

Figure 4. Inflammatory mediator-induced increases in Cl^- currents are dependent on intracellular Ca^{2+} . **A**, IM-induced Cl^- currents were recorded while substituting intracellular EGTA (11 mM) with the more rapid Ca^{2+} chelator, BAPTA (10 mM). The IM-induced increase in Cl^- current was significantly attenuated by 10 mM BAPTA ($n = 7$). Additionally, when Ca^{2+} was buffered to 622 nM with 1.2 mM EGTA in the presence of Cd^{2+} to block influx through Ca^{2+} channels, IMs still produced an increase in inward current at -60 mV ($n = 6$). **B**, To further elucidate the mechanisms of Ca^{2+} -dependent activation, the IM-induced Cl^- current was measured in the presence of ionic solutions in which Cs^+ was used to replace intracellular K^+ and choline was used to replace extracellular Na^+ , leaving Cl^- and Ca^{2+} unchanged. Currents were elicited with a two-pulse protocol, the first to activate Ca^{2+} currents and the second to measure the voltage dependence of activation of Cl^- currents by IMs using digital subtraction. **C**, IM-induced difference currents ($n = 8$) displayed outward rectification and a downward deflection in the I - V curve as the membrane potential was stepped closer to the reversal potential for Ca^{2+} . These currents reversed direction at approximately -30 mV. **D**, Cl^- currents were examined again with Ca^{2+} buffered to 500 nM with 1.2 mM EGTA in the presence of Cd^{2+} to block influx through Ca^{2+} channels. Under these conditions, IM-induced Cl^- currents displayed a linear I - V curve ($n = 7$). Asterisk (*) denotes significant difference between exposures, where $p < 0.05$. Error bars indicate SE.

Figure 5. Inflammatory mediators inhibit voltage-gated Ca^{2+} currents in dural afferents. **A**, IM-induced changes in isolated Ca^{2+} currents were recorded before and then after IM application ($n = 5$). **B**, IM application significantly decreased peak I_{Ca} density. **C**, Instantaneous I - V curves were plotted from tail currents. IMs produced no change in the voltage dependence of activation. *Significant difference between pre-IM and post-IM, where $p < 0.05$. Error bars indicate SE.

induced increase in $[\text{Ca}^{2+}]_{\text{in}}$ was seen in only 41 of 63 nonlabeled neurons. Interestingly, the magnitude of the evoked increase was significantly larger (Fig. 6D,E) and the decay of the transient (in response to a 30 s application of IMs) significantly slower (Fig. 6D,F) in dural afferents than in unlabeled afferents. To rule out the possibility that the augmented Ca^{2+} transients in dural afferents were attributable to DiI labeling, IMs were applied to two additional populations of DiI-labeled afferents: cutaneous afferents retrogradely labeled from the glabrous skin of the hindpaw and muscle afferents retrogradely labeled from the temporalis muscle. Although both populations of afferents can be sensitized by inflammatory mediators (Gold and Traub, 2004; Harriott and Gold, 2009), IM-induced increases in intracellular Ca^{2+} were only observed in 1 of 10 muscle afferents and in 0 of 12 capsaicin-sensitive cutaneous afferents.

To begin to determine whether a combination of inflammatory mediators was actually needed for the sensitization of dural afferents, data were collected from 15 neurons when single mediators were applied sequentially with a 5 min interapplication interval. The order of application was histamine, PGE_2 , and then bradykinin. As with the combination of mediators, an increase of 20% above baseline was considered a response. Of these, six neu-

rons responded to histamine, nine responded to PGE_2 , and five responded to bradykinin. Of these, only three neurons “responsive” to histamine responded to at least one other mediator, whereas the same was true for six neurons responsive to PGE_2 and all five neurons responsive to bradykinin. Although the fraction of neurons responsive to single mediator was not significantly different from the fraction responsive to the combination, the magnitude of the response was smaller ($p < 0.05$) and the decay faster ($p < 0.05$) than was observed when mediators were applied in combination. The increase in fluorescence above baseline was 0.26 ± 0.03 , 0.61 ± 0.18 , and 0.94 ± 0.09 for his-

tamine, PGE_2 , and bradykinin, respectively, whereas T_{50} for decay of the evoked transient was 25.6 ± 6.1 , 65.9 ± 14.2 , and 45.2 ± 10.5 s for histamine, PGE_2 , and bradykinin. These results suggest that the IM-induced increase in $[\text{Ca}^{2+}]_{\text{in}}$ observed in dural afferents reflects the actions of the combination of mediators rather than the actions of any individual mediator.

The role of $I_{\text{IM-Cl}}$ in IM-induced sensitization of dural afferents

In the context of our previous current-clamp data (Harriott and Gold, 2009), the voltage-clamp data presented here suggest that activation of $I_{\text{IM-Cl}}$ is a depolarizing current that increases the excitability of dural afferents. However, these previous data were recorded using whole-cell patch configuration with E_{Cl} (-34 mV) determined by the composition of the bath and electrode solutions. As a result, the “excitatory” effects observed may have been an artifact of our recording conditions. In light of this possibility, it was important to determine whether E_{Cl} in dural afferents was sufficiently depolarized to account for an IM-induced increase in excitability. $I_{\text{IM-Cl}}$ was recorded in response to a ramp protocol from $+50$ to -100 mV using gramicidin perforated patch to prevent dialysis of intracellular Cl^- (Akaike, 1996). To

isolate the current, extracellular TEA (30 mM) was used to block K^+ currents and choline was used to substitute the remaining (100 mM) extracellular Na^+ . Under these recording conditions, I_{IM-Cl} was similar to those recorded under traditional whole-cell patch configuration in Figure 4C (Fig. 7) ($n = 6$). More importantly, results from these experiments indicated that the I_{IM-Cl} reversal potential in dural afferents is -27.73 ± 2.6 mV (Fig. 7B, inset), which is close to the action potential threshold previously recorded in dural afferents (Harriott and Gold, 2009). These observations suggest that activation of I_{IM-Cl} is excitatory in dural afferents.

However, E_{Cl} is generally depolarized relative to the resting membrane potential in sensory neurons, resulting in the phenomenon of primary afferent depolarization first described almost 50 years ago (Eccles et al., 1962). Despite this fact, activation of Cl^- channels via GABA_A receptors, is generally thought to be inhibitory as a result of membrane shunting and depolarization-induced inactivation of voltage-gated Na^+ channels (Price et al., 2009). Therefore, we assessed the impact of IMs on the excitability of dural afferents with gramicidin patch recording (Fig. 8A). Three sets of experiments were performed. In the first, the relative impact of IM-induced activation of I_{IM-Cl} on the sensitization of dural afferents ($n = 7$) was assessed with the application of NFA (100 μ M). Blocking Cl^- channels with NFA alone reduced baseline excitability as indicated by an increase in rheobase (Fig. 8B) and a decrease in the slope of the stimulus response function (SRF) (Fig. 8D), although NFA did not impact the AP threshold (Fig. 8C). These changes were associated with an NFA-induced hyperpolarization of the resting membrane potential. The effects of NFA on rheobase and the SRF (i.e., response to suprathreshold stimulation) returned to baseline after a 2 min washout (Fig. 8B,D,E). Consistent with previous data (Harriott and Gold, 2009), application of IMs produced a significant reduction in rheobase (Fig. 8B), hyperpolarization of AP threshold (Fig. 8C), and increase in the slope of the SRF (Fig. 8D). When NFA was applied in the presence of IMs, NFA reversed the IM-induced decrease in rheobase and the IM-induced increase in the SRF slope (Fig. 8B,D). NFA also reversed the IM-induced membrane depolarization (Fig. 8E). Interestingly, however, NFA did not reverse the hyperpolarization of AP threshold (Fig. 8C), suggesting that, although activation of I_{IM-Cl} is responsible for the most dramatic changes in excitability, it is not responsible for all IM-induced changes.

Figure 6. Inflammatory mediator-induced increases in intracellular Ca^{2+} . **A**, Dural afferents (arrow) and nonlabeled afferents (arrowhead) were identified with epifluorescence illumination. **B**, **C**, Fura-2 fluorescence ratio was low before IM application in both afferent populations (**B**) and dramatically increases in dural afferents and a subpopulation of nonlabeled neurons after IM application (**C**). The IM-induced change in the fluorescence ratio was determined by subtracting the baseline ratio from the peak value. The decay of the IM-induced Ca^{2+} transient was analyzed as time to 50% decay of the peak (T_{50}). **D**, Dural afferents displayed a larger increase in intracellular Ca^{2+} with a slower decay compared with nonlabeled afferents that responded to IMs. **E**, **F**, Of the dural (9) and nonlabeled afferents (41) that responded, the increase in fluorescence was significantly greater in dural afferents than nonlabeled afferents (**E**), and the T_{50} was significantly larger in dural afferents than nonlabeled afferents (**F**). *Significant difference between groups, where $p < 0.05$. Scale bar: (in **A**) **A–C**, 50 μ m. Error bars indicate SE.

Figure 7. The Cl^- equilibrium potential (E_{Cl}) is depolarized in dural afferents. To determine the reversal potential for I_{IM-Cl} , currents were recorded in response to a ramp voltage protocol from +50 to -100 mV using gramicidin perforated patch to prevent dialysis of intracellular Cl^- before and after IM application ($n = 6$). To isolate the current, extracellular TEA was used to block K^+ currents and choline was used to substitute extracellular Na^+ . **A**, Traces demonstrate currents recorded under these conditions before (black trace) and then after (gray trace) IM application. **B**, I_{IM-Cl} obtained with digital subtraction was similar to those recorded in Figure 4C. Additionally, I_{IM-Cl} reversed at -27.73 ± 2.6 mV (inset), which was close to the action potential threshold previously recorded in dural afferents. Error bars indicate SE.

In the second set of experiments, we tested the prediction that, if membrane depolarization associated with a depolarized E_{Cl} underlies IM-induced dural afferent sensitization, hyperpolarization of E_{Cl} should block the sensitizing effects of IMs on dural afferents. To test this prediction, E_{Cl} was artificially hyperpolarized by decreasing the concentration of Cl^- in the electrode solution to 10 mM. Under these recording conditions, the predicted E_{Cl} is -68 mV ($n = 15$). In stark contrast to the 19 of 19 dural afferents sensitized by IMs (Harriott and Gold, 2009) with an E_{Cl}

Figure 8. IM-induced sensitization of dural afferents is blocked by niflumic acid. The impact of I_{IM-Cl} activation was determined with changes in excitability measured in current clamp in the absence and presence of 100 μ M NFA using gramicidin perforated patch to maintain a physiologically relevant E_{Cl} . **Aa**, In the absence of NFA, application of IMs resulted in the sensitization of dural afferents typified by a decrease in rheobase and a leftward shift in the stimulus response function. A typical example of this sensitization is shown in **Aa**, where the protocol used to stimulate the neuron with depolarizing current injection before (baseline) and after application of IMs is shown beneath the voltage traces. Traces evoked at 1, 2, and 3 $\times$ rheobase are shown, with 1.5 and 2.5 $\times$ rheobase omitted for clarity. To enable detection of a leftward shift in the stimulus response function, rheobase was determined both before and after application of IMs so as to appropriately scale the suprathreshold current injection. **Ab**, Voltage traces were evoked in a second neuron before (baseline) and again after the application of NFA (100 μ M), IMs alone, and then the combination of IMs with NFA. Traces evoked after the first wash of NFA have been omitted for clarity. Rheobase determined for each stimulation series is indicated below the voltage traces at 1, 2, and 3 $\times$ rheobase. The resting membrane potential for the neuron in **Aa** was -67 mV, whereas that in **Ab** was -64 mV. Pooled data from seven neurons studied as in **Ab** are plotted in **B–E**. **B**, Blocking Cl^- channels with NFA alone reduced baseline excitability as indicated by an increase in rheobase. This effect returned to baseline levels with a 2 min wash. IMs produced a significant decrease in rheobase compared with wash, which was reversed with subsequent NFA application. **C**, In contrast to the inhibitory NFA effects on rheobase, NFA did not have baseline effects on AP threshold nor was it able to reverse the IM-induced hyperpolarization of AP threshold. **D**, NFA significantly decreased the slope of the SRF, which returned to baseline levels after wash. IMs produced an increase in slope, which was reversed with NFA application. **E**, Consistent with inhibition of a depolarizing current active at rest, NFA alone hyperpolarized the resting membrane potential and reversed the IM-induced depolarization. *Significant difference between exposures, where $p < 0.05$. Error bars indicate SE.

of -34 mV and the 7 of 7 neurons sensitized by IMs with gramicidin patch recording, an E_{Cl} of -68 mV produced an IM-induced decrease in excitability in 5 of 15 dural afferents, as indicated by an increase in rheobase, and produced no detectable change in excitability in an additional 6 of the 15 dural afferents tested. Moreover, an increase in excitability as indicated by a decrease in rheobase was detected in only 4 of 15 dural afferents tested (Fig. 9A). The net effect of IMs in the presence of low intracellular Cl^- was a $15.35 \pm 26.9\%$ increase in rheobase (Fig. 9B) and no changes in the response to suprathreshold stimuli (Fig. 9D). Nevertheless, as with NFA, the IM-induced hyperpolarization of AP threshold was still present in 14 of 15 neurons tested with a hyperpolarized E_{Cl} (Fig. 9C).

In the third set of experiments, we sought to confirm the link between the IM-induced Ca^{2+} transient and the activation of an excitatory Cl^- current. IM-induced sensitization of dural afferents was assessed with an electrode solution in which we again substituted 11 mM EGTA with 10 mM BAPTA ($n = 8$). In the presence of 10 mM BAPTA in the electrode solution, a significant decrease in rheobase was only detected in four of eight neurons

tested. Furthermore, this decrease in rheobase was significantly less than the percentage decrease in rheobase observed in the presence of 11 mM EGTA (Fig. 10A, B). The IM-induced increase in the slope of the SRF was also significantly attenuated in the presence of BAPTA (Fig. 10D). In contrast to the results obtained with NFA and the hyperpolarizing shift in E_{Cl} , 10 mM BAPTA also prevented the IM-induced hyperpolarization of AP threshold (Fig. 10C). These results are consistent with the suggestion that a rapid increase in intracellular Ca^{2+} is necessary for the activation of I_{IM-Cl} and subsequent sensitization of dural afferents and suggest that the Ca^{2+} transient also contributes to the modulation of voltage-gated Na^+ currents critical for the establishment of AP threshold.

Discussion

The purpose of this study was to identify ionic mechanisms underlying IM-induced sensitization of dural afferents. Although at least one experimental outcome, modulation of TTX-R I_{Na} , was expected, our results contain several novel and potentially important observations: (1) the IM-induced block of a Ca^{2+} -dependent K^+

current, (2) the IM-induced inhibition of voltage-gated Ca^{2+} currents, (3) the IM-induced activation of $I_{\text{IM-Cl}}$, and (4) the critical role $I_{\text{IM-Cl}}$ plays in the sensitization of dural afferents.

The IM-induced increase TTX-R I_{Na} in dural afferents observed in the present study is consistent with a growing body of literature indicating that $\text{Na}_v1.8$, the channel underlying the slowly inactivating TTX-R current in nociceptive afferents, is not only a common target for a wide variety of inflammatory mediators but also the dominant channel in nociceptive afferents innervating structures throughout the body (Gold and Caterina, 2008). Results from *in vivo* studies of dural afferents suggest this channel is also present in the peripheral terminals of nociceptive dural afferents (Strassman and Raymond, 1999) where it is positioned to be regulated by inflammatory mediators released in this structure. The absence of detectable IM-induced changes in TTX-S I_{Na} suggests that the IM-induced increase in TTX-R I_{Na} is responsible for increases in AP overshoot and rate of rise (Harriott and Gold, 2009). This increase in Na^+ current is also the most likely mechanism underlying the IM-induced decrease in AP threshold. A unique role for TTX-R I_{Na} in the regulation of AP threshold would account for the observation that the IM-induced hyperpolarization of AP threshold was still observed in the presence of NFA and a hyperpolarized E_{Cl} . Although there is evidence that PGE_2 -induced modulation of TTX-R I_{Na} involves the activation of protein kinase A (PKA) (England et al., 1996; Gold et al., 1998), there is also evidence that PKA activation may be upstream of protein kinase C (PKC) in dissociated neurons as we were able to block PKA-mediated modulation of TTX-R I_{Na} with PKC inhibitors (Gold et al., 1998). The results of the present study are consistent with these previous results if the inhibitor effect of BAPTA reflects the inhibition of IM-induced PKC activation. That is, the inhibition of IM-induced activation of PKC by BAPTA would account for the observation that BAPTA attenuated the IM-induced decrease in AP threshold. However, we suggest that it is the biophysical properties of this channel in combination with the fact that it carries the vast majority of Na^+ current underlying spike initiation in dural afferents that enables activation of $I_{\text{IM-Cl}}$ to have such a profound influence on afferent excitability.

IM-induced suppression of two K^+ currents has been described in sensory neurons. One was a Ca^{2+} -dependent K^+ current underlying a slow afterhyperpolarization in vagal afferents that is blocked by bradykinin and PGE_2 (Cordoba-Rodriguez et al., 1999) that was subsequently described in a small subpopulation of dorsal root ganglion (DRG) neurons (Gold et al., 1996b). These K^+ currents do not appear to be present in dural afferents. The second, a K^+ current suppressed by inflammatory mediators including PGE_2 , is a α -dendrotoxin-sensitive current, likely mediated by a K^+ channel with $\text{Kv}1.1$ properties (Chi and Nicol, 2007). This current has a

Figure 9. IM-induced sensitization of dural afferent is blocked with a hyperpolarizing shift in E_{Cl} . IM-induced changes in dural afferent excitability was measured in the presence of 10 mM intracellular Cl^- to hyperpolarize the reversal potential for Cl^- from -34 to -68 mV ($n = 15$). **A**, Changing the E_{Cl} to -68 mV produced an IM-induced increase in rheobase in 5 of 15 dural afferents, produced no effect in 6 of 15 dural afferents, and decreased rheobase in only 4 of 15 dural afferents. **B**, The net effect of IMs in the presence of low intracellular Cl^- was a $15.35 \pm 26.9\%$ increase in rheobase, which was significantly different from that observed with an E_{Cl} of -34 mV. **C**, In contrast to these results, IMs were still able to significantly hyperpolarize AP threshold with an E_{Cl} of -68 mV. **D**, However, with an E_{Cl} of -68 mV, IMs did not shift the response to suprathreshold stimulation. Asterisk (*) denotes significant difference between exposures, where $p < 0.05$. Error bars indicate SE.

Figure 10. IM-induced sensitization of dural afferent is blocked by BAPTA. Increases in excitability were examined after substituting 11 mM EGTA for 10 mM BAPTA ($n = 8$) to determine whether, similar to $I_{\text{IM-Cl}}$, IM-induced sensitization was sensitive to rapid Ca^{2+} chelation. **A**, In the presence of 10 mM BAPTA, four of eight cells exhibited a reduction in rheobase. **B**, On average, there was a reduction in rheobase in the presence of IMs; however, consistent with a role for intracellular Ca^{2+} , this reduction was significantly less than the reduction observed in the presence of 11 mM EGTA. **C**, The 10 mM BAPTA prevented the IM-induced hyperpolarization of AP threshold. **D**, Additionally, although there was a left shift in the SRF, there was no change in the slope. *Significant difference between pre-IM and post-IM, where $p < 0.05$. Error bars indicate SE.

relatively low threshold for activation and contributes to a decrease in current threshold, action potential threshold, and the increase in burst duration in capsaicin-sensitive small-diameter DRG neurons. Despite the inclusion of PGE_2 in the IMs used in the present study, no such current was suppressed in dural afferents. Instead, IMs suppressed an ibuprofen-insensitive Ca^{2+} -dependent K^+ current. Given the biophysical properties of the IM-sensitive K^+ current in dural afferents, it is unlikely to contribute to the changes in excitability measured here but may contribute more readily after prolonged stimuli in which it may begin to play a more dominant role as other K^+ currents begin to inactivate.

The efficacy of BAPTA to block both the activation of $I_{\text{IM-Cl}}$ and the IM-induced sensitization of dural afferents suggests that activation of $I_{\text{IM-Cl}}$ is dependent on a transient increase in $[\text{Ca}^{2+}]_{\text{in}}$. Whether it is the rapid IM-induced increase in $[\text{Ca}^{2+}]_{\text{in}}$ that is essential for the activation of $I_{\text{IM-Cl}}$, or that activation of this current requires the rise in Ca^{2+} coincident to additional second messenger pathway(s) activated by IMs, remains to be determined. Nevertheless, what is clear is that an increase in $[\text{Ca}^{2+}]_{\text{in}}$ alone, is insufficient for the activation of $I_{\text{IM-Cl}}$. BAPTA will only influence the kinetics of the Ca^{2+} transient without significantly attenuating the magnitude or duration of the IM-induced increase in $[\text{Ca}^{2+}]_{\text{in}}$. Furthermore, neither the

increase in $[Ca^{2+}]_{in}$ alone via the activation of voltage-gated Ca^{2+} currents or artificially buffering $[Ca^{2+}]_{in}$ to >600 nM was sufficient to activate a Cl^{-} current in dural afferents. Our data also suggest that, once activated, I_{IM-Cl} is not influenced by additional increases in $[Ca^{2+}]_{in}$. That is, given IM-induced suppression of voltage-gated Ca^{2+} currents, a decrease in an inward current (i.e., Ca^{2+}) at potentials greater than -30 mV (see below) is likely to account for odd shape of the I_{IM-Cl} I - V curve (i.e., Fig. 4C), rather than an influence of Ca^{2+} that is selective for Cl^{-} influx.

Recent data suggest that after nerve injury, expression of the Ca^{2+} -dependent Cl^{-} channel, bestrophin-1, is upregulated in DRG neurons (Boudes et al., 2009), which may be responsible for the nerve injury-induced increase in Ca^{2+} -dependent Cl^{-} current in DRG (André et al., 2003; Boudes et al., 2009) and nodose ganglion (Lancaster et al., 2002) neurons. Such a channel may also account for the Cl^{-} current activated by bradykinin in vagal afferents (Oh and Weinreich, 2004; Lee et al., 2005). However, this is the first description of a Cl^{-} current contributing to the IM-induced sensitization of somatic afferents. Furthermore, the IM-activated Cl^{-} current in dural afferents appears to be distinct from Cl^{-} channels upregulated after nerve injury, and bestrophin-1, given that these channels are readily activated by any increase in $[Ca^{2+}]_{in}$, such as that associated with action potential generation (Boudes et al., 2009), whereas I_{IM-Cl} , once activated, appears to be mostly Ca^{2+} independent.

Suppression of voltage-gated Ca^{2+} currents in sensory neurons is generally believed to be one of the primary mechanisms underlying the actions of several spinally administered analgesics (Gold and Caterina, 2008). Nevertheless, PGE_2 -induced suppression of Ca^{2+} currents in mouse trigeminal ganglion neurons has been described previously (Borgland et al., 2002). It is therefore possible that the IM-induced suppression of Ca^{2+} current in dural afferents reflects a similar mechanism of action. However, in contrast to the results obtained with PGE_2 in mouse trigeminal ganglion neurons, suppression of Ca^{2+} currents in dural afferents did not appear to reflect a membrane delimited binding of G-protein subunits that would be expected to shift the voltage dependence of channel activation. Alternatively, the large sustained IM-induced increases in $[Ca^{2+}]_{in}$ raise the possibility that inhibition of Ca^{2+} currents in dural afferents is mediated by a Ca^{2+} -induced inactivation of Ca^{2+} channels (Catterall, 2000).

IM-induced suppression of Ca^{2+} currents in dural afferents may serve as a form of a “brake” to the excitatory IM-induced processes, for example by attenuating the peripheral release of transmitters from nociceptive afferents and, consequently, the neurogenic inflammation mediated by vasoactive neuropeptides in dural afferents (McIlvried et al., 2009). However, a decrease in voltage-gated Ca^{2+} currents may also contribute indirectly to dural afferent sensitization, as the IM-induced decrease in Ca^{2+} current is likely to be responsible for the IM-induced decrease in high-threshold Ca^{2+} -dependent K^{+} current.

The most dramatic IM-induced changes in dural afferent excitability are the decrease in rheobase and the shift in the stimulus response function. That IM-induced activation of I_{IM-Cl} plays a dominant role in both of these changes is suggested by the observation that both changes were reversed by NFA, attenuated by BAPTA, and switched from being excitatory to inhibitory when E_{Cl} was hyperpolarized. These observations suggest that the mix of ion channels underlying the excitability of dural afferents is unique from those regulating the excitability of other populations of afferents in which the membrane depolarization associated with the activation of Cl^{-} channels is inhibitory (Price et al., 2009). That I_{IM-Cl} does not appear to contribute to the sensitiza-

tion of other populations of afferents we have studied (Gold and Traub, 2004; Harriott and Gold, 2009) does not rule out the possibility that the channel contributes to the sensitization of other afferent populations, but it does raise the intriguing possibility that it may be unique to dural afferents.

Although the results of the present study have added several important aspects to our understanding of dural afferents, several caveats must be kept in mind. First, although several lines of evidence suggest that the impact of dural afferent labeling on the properties of dural afferents should be minimal [mast cell degranulation normalized by 10 d after labeling (Harriott and Gold, 2009); the IM-induced changes in excitability are comparable with those observed in unlabeled sensory neurons (Gold et al., 1996c); and the IM-induced sensitization of other populations of trigeminal ganglion neurons (Harriott and Gold, 2009), even those such as pulpal afferents that require significant tissue destruction to enable retrograde labeling (our unpublished observation), still appears to involve processes distinct from those observed in dural afferents], it is possible that the process of cell labeling has changed the properties of dural afferents. Second, despite evidence from other afferent populations suggesting that mechanisms underlying the actions of inflammatory mediators, as revealed through the study of the afferent cell body *in vitro* (Gold et al., 1996a), contribute to the sensitization of afferent terminals *in vivo* (Khasar et al., 1998), the afferent cell body *in vitro* is only a model of the afferent terminal. With a number of potentially important limitations (i.e., injury, differences in anatomical constraints, etc) associated with this model, additional experiments will be necessary to confirm the contribution of processes identified in the present study to the sensitization of dural afferent terminals *in vivo*.

Although there is evidence that central mechanisms are involved in triggering migraine, dural afferents appear to be critical for the initiation of migraine pain (Moskowitz et al., 1993; Strassman et al., 1996; Burstein, 2001; Bolay and Moskowitz, 2002). Activating the peripheral terminals of dural afferents produces pain that is identical with a migraine (Ray and Wolff, 1940). Furthermore, there is evidence that neurogenic inflammation of the dura can activate and sensitize this population of afferents during a migraine attack (Strassman et al., 1996; Sarchielli et al., 2000). The data presented here suggest ionic mechanisms that may serve as targets for the development of novel antimigraine therapies. The fact that $Na_v1.8$ is present in all dural afferents in which it appears to play the dominant role in spike initiation (Strassman and Raymond, 1999) supports the notion that a selective blocker would not only help other types of pain but may be effective for migraine pain as well. The critical role of I_{IM-Cl} in mediating IM-induced sensitization of dural afferents suggest that the ion channel underlying this current and/or the mechanisms underlying the maintenance of the Cl^{-} gradients in dural afferent may be a particularly useful target for the treatment of migraine.

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