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The Kv4.2 Potassium Channel Subunit Is Required for Pain Plasticity

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Summary

A-type potassium currents are important determinants of neuronal excitability. In spinal cord dorsal horn neurons, A-type currents are modulated by extracellular signal-regulated kinases (ERKs), which mediate central sensitization during inflammatory pain. Here, we report that Kv4.2 mediates the majority of A-type current in dorsal horn neurons and is a critical site for modulation of neuronal excitability and nociceptive behaviors. Genetic elimination of Kv4.2 reduces A-type currents and increases excitability of dorsal horn neurons, resulting in enhanced sensitivity to tactile and thermal stimuli. Furthermore, ERK-mediated modulation of excitability in dorsal horn neurons and ERK-dependent forms of pain hypersensitivity are absent in *Kv4.2*$^{-/-}$ mice compared to wild-type littermates. Finally, mutational analysis of Kv4.2 indicates that S616 is the functionally relevant ERK phosphorylation site for modulation of Kv4.2-mediated currents in neurons. These results show that Kv4.2 is a downstream target of ERK in spinal cord and plays a crucial role in pain plasticity.

Introduction

Chronic pain is an expression of neuronal plasticity, which is mediated in part by increased excitability of nociceptive neurons in the dorsal horn of the spinal cord (Ji and Woolf, 2001). The molecular mechanisms that underlie this nociceptive plasticity are not fully understood, but the extracellular signal-regulated kinases (ERKs) have been implicated in the development of spinal central sensitization underlying persistent pain (Adwanikar et al., 2004; Galan et al., 2002; Hu and Gereau, 2003; Hu et al., 2003; Ji et al., 1999, 2002, 2003; Ji and Woolf, 2001; Karim et al., 2001, 2006; Kawasaki et al., 2004; Kominato et al., 2003; Lever et al., 2003; Pezet et al., 2002). Although the precise cellular mechanisms of ERK-dependent central sensitization are not known, a number of mechanisms have been proposed, including regulation of gene transcription and phosphorylation-dependent modulation of ion channels.

Among the potential ion channel targets, transient outward (A-type) potassium channels have emerged as attractive candidate sites of modulation for ERK-dependent central sensitization of spinal cord dorsal horn neurons (Hu and Gereau, 2003; Hu et al., 2003; Ji et al., 2003; Karim et al., 2006). A-type K+ channels activate at subthreshold membrane potentials, inactivate rapidly, and rapidly recover from inactivation. A-type currents are important regulators of neuronal excitability and have been implicated in synaptic plasticity (Ramakers and Storm, 2002; Watanabe et al., 2002). In the brain, A-type currents can be generated by Kv1.4, Kv3.4, or any of the Kv4 family subunits (Kv4.1, Kv4.2, and Kv4.3) (Pongs, 1999; Song, 2002). ERK activation decreases A-type currents and increases excitability of neurons in the superficial spinal cord dorsal horn (Hu and Gereau, 2003; Hu et al., 2003; Karim et al., 2006) and hippocampus (Watanabe et al., 2002; Yuan et al., 2002). Furthermore, previous studies have demonstrated that Kv4.2 is directly phosphorylated by ERKs in vitro and in vivo (Adams et al., 2000). Since ERKs play important roles both in nociception and modulation of A-type currents, we hypothesized that the Kv4.2 subunit might be a downstream target of ERK in dorsal horn neurons that contributes to A-type currents and modulates neuronal excitability and nociceptive behavior. Because of the dearth of pharmacologic reagents that target Kv4 channels, we have utilized dominant-negative constructs and Kv4.2 knockout mice to directly test this hypothesis.

Results

Kv4 Channels Underlie A-Type K+ Currents in Dorsal Horn Neurons

Kv4 channels are products of three distinct genes: Kv4.1, Kv4.2, and Kv4.3 (Coetzee et al., 1999; Jerng et al., 2004). To test whether these Kv4 subunits contribute to A-type currents in dorsal horn neurons, mouse spinal cord superficial dorsal horn neurons were transfected with an EGFP-tagged dominant-negative Kv4.2 construct (Kv4.2dn) containing a single amino acid substitution (W362F) (Barry et al., 1998) or with EGFP alone. Using whole-cell voltage-clamp recordings in neurons transfected with EGFP, large outward currents were evoked by a step depolarization from a holding potential of −80 mV to +40 mV (Figure 1A), similar to what we have reported previously in untransfected neurons (Hu et al., 2003). Transfection with EGFP-Kv4.2dn dramatically reduced the A-type current (Figure 1A). Average A-type current density in EGFP-Kv4.2dn-transfected neurons was decreased by 79% compared to EGFP-transfected controls, while sustained currents were not affected (Figure 1B). Because the Kv4.2dn construct will disrupt

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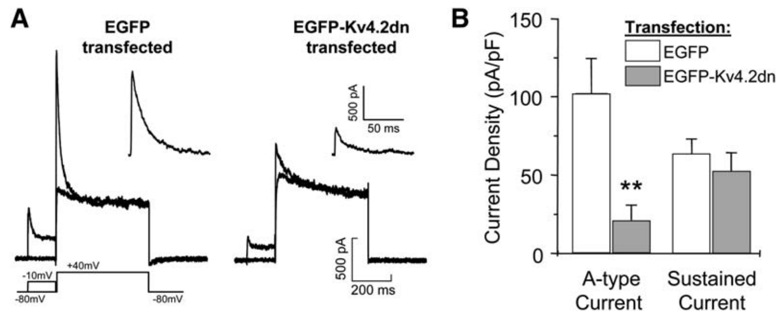

Figure 1. Kv4 Subunits Mediate A-Type K+ Currents in Spinal Cord Dorsal Horn Neurons

(A) Outward potassium currents recorded in cultured mouse superficial dorsal horn neurons transfected with control (EGFP, left panel) or Kv4.2 dominant-negative (EGFP-Kv4.2dn, right panel) constructs. The insets show A-type currents after offline subtraction of the sustained currents.

(B) Mean densities of A-type currents and sustained currents in EGFP- or EGFP-Kv4.2dn-transfected neurons. Values represent mean ± SEM; n = 12–16 neurons. **$p < 0.01$.

channels containing any Kv4 subunit (Barry et al., 1998), these results suggest that the Kv4 family is a prominent contributor of A-type currents in dorsal horn neurons. Consistent with this, immunostaining revealed strong staining in the superficial layers of the spinal cord dorsal horn using both anti-Kv4.2 and anti-Kv4.3 antibodies (Figures 2B and 2C).

Kv4.2 Knockout Mice Reveal a Central Role of Kv4.2 in Regulating Neuronal Excitability of Spinal Dorsal Horn Neurons

To test the hypothesis that Kv4.2 contributes to A-type currents in superficial dorsal horn neurons, we examined A-type currents in dorsal horn neurons from mice carrying a null mutation in the *Kv4.2* gene that we have generated by homologous recombination (Guo et al., 2005). In the targeting construct, the principal exon, which encodes most of the Kv4.2 channel (from the start codon to Gly-373 in the middle of the pore domain), was replaced with a neomycin cassette to create a null allele that would neither give rise to a functional channel nor produce a defective subunit that would dominantly interfere with other Kv4 subunits. The lack of Kv4.2 protein expression in the spinal cord of *Kv4.2*$^{-/-}$ mice was verified by immunoblotting and immunocytochemistry using an anti-Kv4.2 antibody (Figures 2A and 2B). Kv4.3 protein was detected in both wild-type and *Kv4.2*$^{-/-}$ mice (Figure 2C).

To investigate whether deletion of Kv4.2 alters A-type currents, potassium currents were recorded from superficial dorsal horn neurons prepared from wt or *Kv4.2*$^{-/-}$ mice. Similar to what was seen in wt neurons transfected with the Kv4.2dn construct, the amplitude of A-type currents was decreased by 56% in *Kv4.2*$^{-/-}$ neurons compared to parallel cultures prepared from wt mice, while sustained currents did not significantly differ between genotypes (Figures 2D and 2E). In *Kv4.2*$^{-/-}$ neurons, the activation curve for A-type currents is significantly shifted to the left by 6.5 mV with no change in slope relative to wt (Figure 2F). The voltage of half-maximal

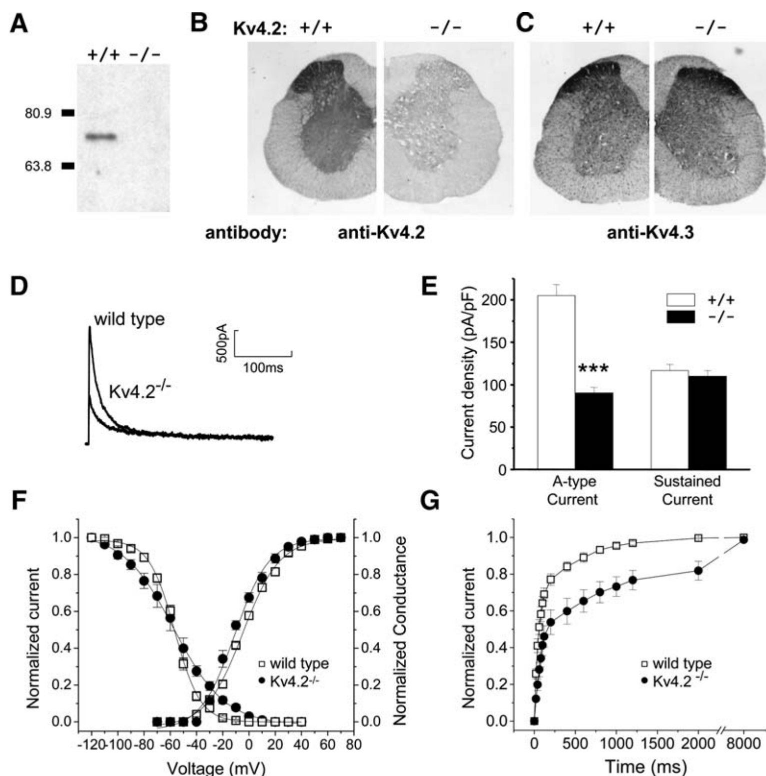

Figure 2. Genetic Elimination of Kv4.2 Dramatically Alters A-Type Currents in Dorsal Horn Neurons

(A) Immunoblot of Kv4.2 protein from wt or *Kv4.2*$^{-/-}$ spinal cords. The position of molecular weight markers is indicated.

(B and C) Immunohistochemical staining of lumbar spinal cord sections from wt (left panels) and *Kv4.2*$^{-/-}$ mice (right panels) with anti-Kv4.2 (B) or anti-Kv4.3 (C) antibodies.

(D) Representative A-type currents recorded from wt or *Kv4.2*$^{-/-}$ neuronal cultures.

(E) Mean densities of A-type currents and sustained currents in wt (n = 30) or *Kv4.2*$^{-/-}$ (n = 44) neurons. Values represent mean ± SEM; ***$p < 0.001$.

(F) Steady-state inactivation and activation curves from wt (n = 7) or *Kv4.2*$^{-/-}$ neurons (n = 9).

(G) Recovery from inactivation of A-type currents in wt (n = 7) or *Kv4.2*$^{-/-}$ (n = 8) neurons.

inactivation was not significantly different between the two genotypes, but the slope of the steady-state inactivation curve in neurons from *Kv4.2*$^{-/-}$ neurons (19.5 ± 2.0) was significantly different from that in wt neurons (9.7 ± 0.9, Figure 2F; $p < 0.05$, ANOVA). In addition, recovery of A-type currents from inactivation was significantly slower in *Kv4.2*$^{-/-}$ neurons than in wt (Figure 2G). These changes in functional properties of A-type currents in *Kv4.2*$^{-/-}$ mice were rescued by transfection of dorsal horn neurons from *Kv4.2*$^{-/-}$ mice with a wild-type Kv4.2 construct (Figures 3A–3C), indicating that the decrease in A-type currents observed in dorsal horn neurons from *Kv4.2*$^{-/-}$ mice is due to loss of Kv4.2 expression as opposed to an associated compensatory change in expression of another gene. These results suggest that Kv4.2 expression is necessary for the majority of A-type currents in mouse superficial dorsal horn neurons.

Our previous studies showed that acute decreases in A-type currents cause increases in neuronal excitability in dorsal horn neurons (Hu and Gereau, 2003). As deletion of Kv4.2 also reduces A-type currents relative to wild-type in dorsal horn neurons, we hypothesized that neuronal excitability would be enhanced in dorsal horn neurons from *Kv4.2*$^{-/-}$ mice relative to those from wild-type mice. To test this hypothesis, we performed whole-cell current-clamp recordings in superficial dorsal horn neurons from spinal cord slices prepared from wt or *Kv4.2*$^{-/-}$ mice. Lamina I/II neurons from *Kv4.2*$^{-/-}$ mice showed dramatically enhanced excitability compared to neurons in slices from wt mice. In slices from wt mice, the resting membrane potential was −66.4 ± 1.6 mV (n = 28) and the mean rheobase (the minimum current required to discharge an action potential) was 9.5 ± 1.0 pA. Deletion of Kv4.2 significantly changed the membrane potential to −62.2 ± 1.2 mV (n = 25, $p < 0.05$) and significantly decreased the rheobase to 5.5 ± 0.6 pA (Figure 4). During rheobase measurements, neurons were always held at −70 mV via current injection. These results indicate that Kv4.2-mediated A-type currents regulate neuronal excitability in spinal cord superficial dorsal horn neurons.

We and others have previously identified at least four categories of superficial dorsal horn neurons based on firing properties in culture and in slices (Hu and Gereau, 2003; Ruscheweyh and Sandkuhler, 2002). Similarly, in the present study we identified neurons in spinal cord slices from ICR mice or wild-type 129SvEv mice that include a mixture of repetitive (tonic), phasic, and delayed firing patterns. Interestingly, *Kv4.2*$^{-/-}$ mice show a change in the distribution, with a near absence of phasic firing cells and a coincident increase in the group of cells showing repetitive firing in response to a step current injection (Table 1). This is similar to previous results obtained in superior cervical ganglion neurons transfected with a Kv4.2 dominant-negative construct (Malin and Nerbonne, 2001) and suggest that Kv4.2-containing channels may contribute to the currents that mediate spike frequency adaptation in dorsal horn neurons. Alternatively, the absence of Kv4.2 may lead to compensatory changes in other ion channels that normally generate this phasic firing pattern in dorsal horn neurons. The increase in the percentage of tonic-firing neurons suggests that the neurons that have a phasic firing pattern in wild-type animals have been converted to a tonic-firing pattern in *Kv4.2*$^{-/-}$ mice. The precise mechanisms of this change are not known.

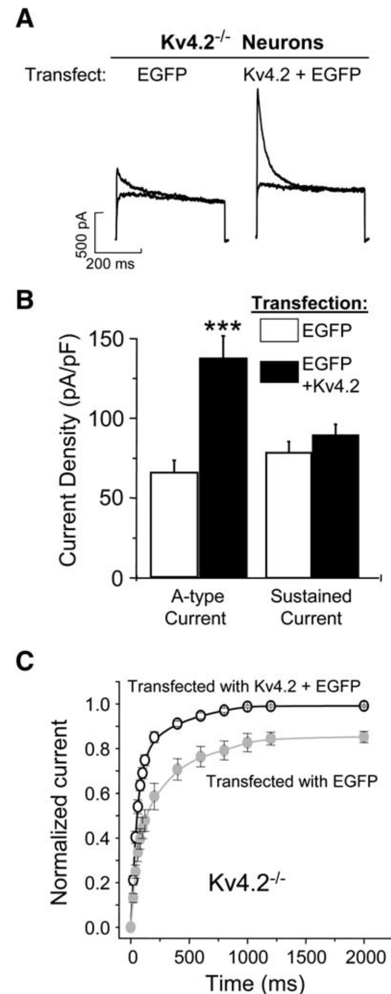

Figure 3. Rescue of A-Type Currents in *Kv4.2*$^{-/-}$ Neurons by Transfection with a Wild-Type Kv4.2 Construct

(A) Outward potassium currents recorded in *Kv4.2*$^{-/-}$ neurons transfected with EGFP or EGFP + Kv4.2.

(B) Mean densities of A-type currents and sustained currents in *Kv4.2*$^{-/-}$ neurons transfected with EGFP (n = 20) or EGFP + Kv4.2 (n = 22). Values represent mean ± SEM; ***$p < 0.001$.

(C) Recovery from inactivation of A-type currents recorded in *Kv4.2*$^{-/-}$ neurons transfected with EGFP (n = 6) or EGFP + Kv4.2 (n = 8).

Altered Nociception in Kv4.2 Knockout Mice

The results described above demonstrate that Kv4.2 underlies the majority of A-type current in spinal dorsal horn neurons, and accordingly dorsal horn neurons from *Kv4.2*$^{-/-}$ mice show increased excitability relative to neurons from wt mice (Figure 4). Because superficial dorsal horn neurons are involved in nociceptive transmission, we hypothesized that the altered neuronal excitability in *Kv4.2*$^{-/-}$ mice would result in altered pain transmission in these animals. To test this hypothesis, we evaluated nociceptive behavior in *Kv4.2*$^{-/-}$ mice and their wt littermates (*Kv4.2*$^{+/+}$). All behavioral studies

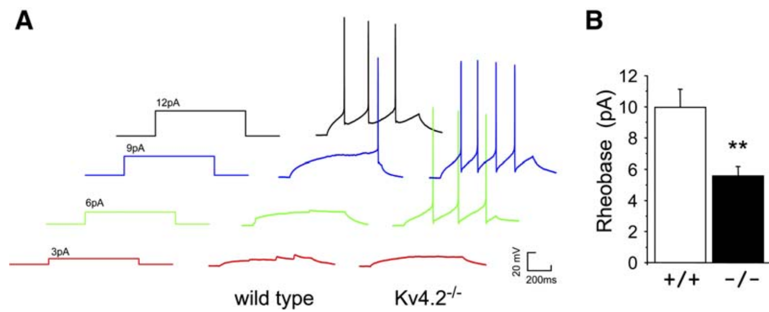

Figure 4. Genetic Elimination of Kv4.2 Results in Increased Excitability of Dorsal Horn Neurons

(A) Representative action potentials generated by an increasing series of current injections (left panel) recorded in superficial (lamina I-II) dorsal horn neurons in spinal cord slices prepared from wt (center panel) or *Kv4.2*$^{-/-}$ (right panel) mice.

(B) Rheobase (the minimum current required to elicit an action potential) in neurons from wt (n = 28) or *Kv4.2*$^{-/-}$ (n = 25) mice. For rheobase measurements, neurons were always held at −70 mV via current injection. Values represent mean ± SEM; **p < 0.01.

were performed prior to genotyping, and thus the experimenter was always blind to genotype. *Kv4.2*$^{-/-}$ mice were indistinguishable from sex- and age-matched wild-type littermates in appearance, general behavior, and body weight (data not shown) (Guo et al., 2005). Furthermore, motor function assessed using an accelerating rotarod was normal (Figure 5A). However, consistent with our hypothesis, *Kv4.2*$^{-/-}$ mice displayed significantly enhanced sensitivity to mechanical and thermal stimuli compared to *Kv4.2*$^{+/+}$ littermates. The paw-withdrawal threshold to von Frey filament stimulation and the response threshold to noxious pressure applied to the tail were significantly reduced in *Kv4.2*$^{-/-}$ mice compared to their *Kv4.2*$^{+/+}$ littermates (Figures 5B and 5C). *Kv4.2*$^{-/-}$ mice also had significantly shorter withdrawal latencies to noxious heat in the hotplate test when compared to their *Kv4.2*$^{+/+}$ littermates (Figure 5D).

ERK-Dependent Modulation of Dorsal Horn Neuronal Excitability Requires Kv4.2

Our previous studies showed that activation of ERK causes significant inhibition of A-type currents and accordingly increases neuronal excitability in mouse dorsal horn neurons (Hu and Gereau, 2003; Hu et al., 2003). Given that the intracellular C terminus of Kv4.2 can serve as an ERK substrate in vitro (Adams et al., 2000), we reasoned that the ERK-dependent modulation of A-type currents and neuronal excitability in dorsal horn neurons might require Kv4.2 expression. To test this hypothesis, we compared the effects of ERK activation and inhibition in dorsal horn neurons from wt and *Kv4.2*$^{-/-}$ mice.

There are no specific pharmacologic agents that activate the ERK pathway independent of other kinases. However, activation of PKA or PKC can lead to downstream activation of MEK/ERK signaling. We previously showed that all of the effects of the PKC activator PMA on dorsal horn neuronal physiology are blocked by the MEK inhibitors U0126 and PD98059, suggesting that these effects of PMA are due to activation of ERK downstream of PKC (Hu and Gereau, 2003; Hu et al., 2003). We therefore tested the effects of PMA (5 μM) as an upstream activator of ERK, or the MEK inhibitor PD98059 (20 μM), which inhibits activation of ERK, on cultured dorsal horn neurons or lamina I/II neurons in spinal cord slices. Consistent with our previous report (Hu et al., 2003), in cultured dorsal horn neurons from wt mice, PMA significantly reduced A-type current amplitude, while PD98059 increased the current amplitude. In the *Kv4.2*$^{-/-}$ neurons, however, PMA had no effect on A-type currents, and PD98059 did not increase but rather decreased A-type currents (Figures 6A and 6B). These results demonstrate that the inhibition of A-type currents by PMA and the PD98059-induced increases in these currents require expression of the Kv4.2 subunit. The inhibitory effect of PD98059 on A-type currents in *Kv4.2*$^{-/-}$ neurons compared to the enhancement of A-type currents by PD98059 in wt neurons could be due to an inhibitory modulation by ERK signaling of the residual current in the *Kv4.2*$^{-/-}$ neurons or could represent a nonspecific effect of the drug.

In current-clamp recordings in slices prepared from wt mice, PMA significantly decreased first-spike latency and increased spike frequency, while PD98059 significantly increased the first-spike latency and reduced the spike frequency. These effects of ERK activation by PMA, or inhibition by PD98059, were completely absent in slices prepared from *Kv4.2*$^{-/-}$ mice (Figures 6C and 6D). These results indicate that expression of the Kv4.2 subunit is necessary for ERK-dependent modulation of neuronal excitability in spinal cord dorsal horn neurons.

Loss of ERK-Dependent Hyperalgesia in Kv4.2 Knockout Mice

The behavioral data to this point showing hypersensitivity to tactile and thermal stimuli are completely consistent with our observations of enhanced excitability of dorsal horn neurons in the *Kv4.2*$^{-/-}$ mice. A further prediction, based on our observation that ERK-dependent modulation of A-type currents and neuronal excitability in dorsal horn neurons requires Kv4.2 expression, is

Table 1. Firing Properties of Dorsal Horn Neurons from Spinal Cord Slices

Mouse	Repetitive	Phasic	Delayed Firing	Single Spike
ICR	28/56 (50%)	14/56 (25%)	12/56 (21%)	2/56 (4%)
Wild-type 129SvEv	16/28 (57%)	7/28 (25%)	4/28 (14%)	1/28 (4%)
Kv4.2$^{-/-}$ 129SvEv	26/30 (87%)	1/30 (3%)	3/30 (10%)	0/30 (0%)

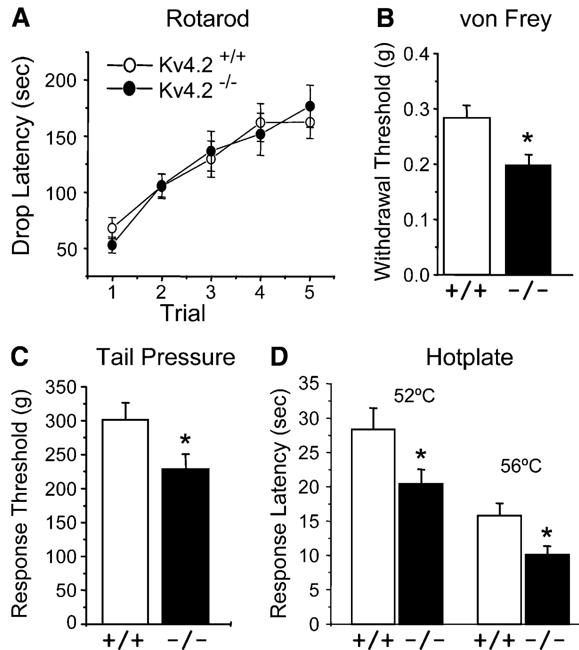

Figure 5. Enhanced Basal Pain Behavioral Responses in *Kv4.2*$^{-/-}$ Mice

All values represent mean ± SEM.

(A) Drop latencies on an accelerating rotarod (n = 14–16).

(B) Paw-withdrawal thresholds to von Frey filaments (n = 8–11).

(C) Response threshold to tail pressure (n = 14–16).

(D) Response latencies in the hot-plate test at 52ºC or 56ºC (n = 14–16).

*$p < 0.05$, versus wild-type, ANOVA. All mice used for behavioral studies in this figure were on the 129 genetic background.

that ERK-dependent forms of behavioral sensitization involving central sensitization of dorsal horn neurons should also be absent in these animals. The prediction is that the *Kv4.2*$^{-/-}$ mice, while hypersensitive in the basal condition due to increased excitability of dorsal horn neurons, would not develop further hypersensitivity that results from ERK-dependent modulation in dorsal horn neurons. To test this hypothesis, we compared *Kv4.2*$^{-/-}$ mice to wild-type littermates in the formalin and carrageenan models of inflammatory pain and in the chronic constriction injury (CCI) model of neuropathic pain, all of which have been shown to involve ERK activation in the spinal dorsal horn (Ciruela et al., 2003; Galan et al., 2002; Ji et al., 1999; Karim et al., 2001; Song et al., 2005).

The formalin test is commonly employed as a test of inflammatory pain in rodents. Injection of formalin into the hindpaw activates nociceptors and results in a typical biphasic nociceptive response (Karim et al., 2001). The first phase of nocifensive behaviors is generally believed to involve direct activation of nociceptors, whereas the second phase is believed to additionally involve peripheral and central sensitization (Puig and Sorkin, 1996). This sensitization in the second phase has been shown by multiple groups to require ERK activation in the spinal dorsal horn (Ji et al., 1999; Karim et al., 2001). We therefore compared the response of *Kv4.2*$^{-/-}$ mice and *Kv4.2*$^{+/+}$ littermates in the formalin test. For this experiment, we utilized mice backcrossed onto the FVB background for >12 generations, as the 129SvEv strain of mice expressed very little formalin-induced behaviors in the second phase (data not shown), consistent with previous reports (Mogil et al., 1999). No significant difference between *Kv4.2*$^{-/-}$ mice and *Kv4.2*$^{+/+}$ littermates was detected in the acute phase (Figure 7A), but the second phase behavioral response in the *Kv4.2*$^{-/-}$ mice is very interesting. These mice show a constant mild elevated response compared to the *Kv4.2*$^{+/+}$ mice; however, they lack the peak second phase response observed in the *Kv4.2*$^{+/+}$ littermates (Figure 7A). While an ANOVA comparing the traditionally defined formalin second phase (15 min to 60 min post-formalin) did not reach significance, a significant difference is observed between *Kv4.2*$^{-/-}$ mice and *Kv4.2*$^{+/+}$ littermates when considering nociceptive behaviors 10–30 min after formalin injection (repeated-measures ANOVA). There is no significant difference between genotypes for the 35–45 min block. The constant mild elevated second phase response observed in *Kv4.2*$^{-/-}$ mice is consistent with the idea that there is enhanced postsynaptic firing (due to enhanced excitability of dorsal horn neurons in *Kv4.2*$^{-/-}$ animals) in response to the low-level barrage of C fiber input associated with the formalin second phase (Puig and Sorkin, 1996), whereas the lack of a peak second phase in the *Kv4.2*$^{-/-}$ mice can be attributed to the lack of ERK-dependent modulation of excitability in these superficial dorsal horn neurons. Interestingly, these results in the formalin test are reminiscent of our results of studies of transgenic mice that express dominant-negative MEK in neurons, in which we observed a reduction in only the first half of the second phase of the formalin test (Karim et al., 2006).

We next tested for the presence of mechanical hypersensitivity 1–3 hr following formalin injections. *Kv4.2*$^{+/+}$ mice developed significant ipsilateral and contralateral hypersensitivity to mechanical stimuli, while *Kv4.2*$^{-/-}$ littermates were not hypersensitive relative to baseline mechanical thresholds (Figure 7B), suggesting that Kv4.2 is required for inflammation-induced mechanical hypersensitivity. While previous studies have reported enhanced nociceptive sensitivity following formalin-induced inflammation (Fu et al., 2000, 2001; Zeitz et al., 2004), no studies have addressed whether this hypersensitivity is dependent on spinal ERK signaling. We therefore investigated whether the absence of mechanical hypersensitivity in *Kv4.2*$^{-/-}$ mice was due to a lack of ERK-dependent modulation in the spinal cord. We pretreated wt mice with the MEK inhibitor U0126 (2 nmol, intrathecal) for 15 min, then injected formalin into the hindpaw. U0126 application did not alter basal mechanical thresholds, but significantly reduced formalin-induced mechanical hypersensitivity (Figures 7C and 7D). These results suggest that tactile hypersensitivity after formalin-induced inflammation requires ERK activation in the spinal cord, and further implicates ERK-mediated modulation of Kv4.2-containing channels in this process. It is important to point out that we used mice from several different genetic backgrounds (129SvEv, FVB, and ICR) in this study. Although the absolute mechanical withdrawal thresholds were different between the strains, the *Kv4.2*$^{-/-}$ mice were consistently more sensitive to touch at baseline than wild-type littermates for a given strain (compare 129 mice in

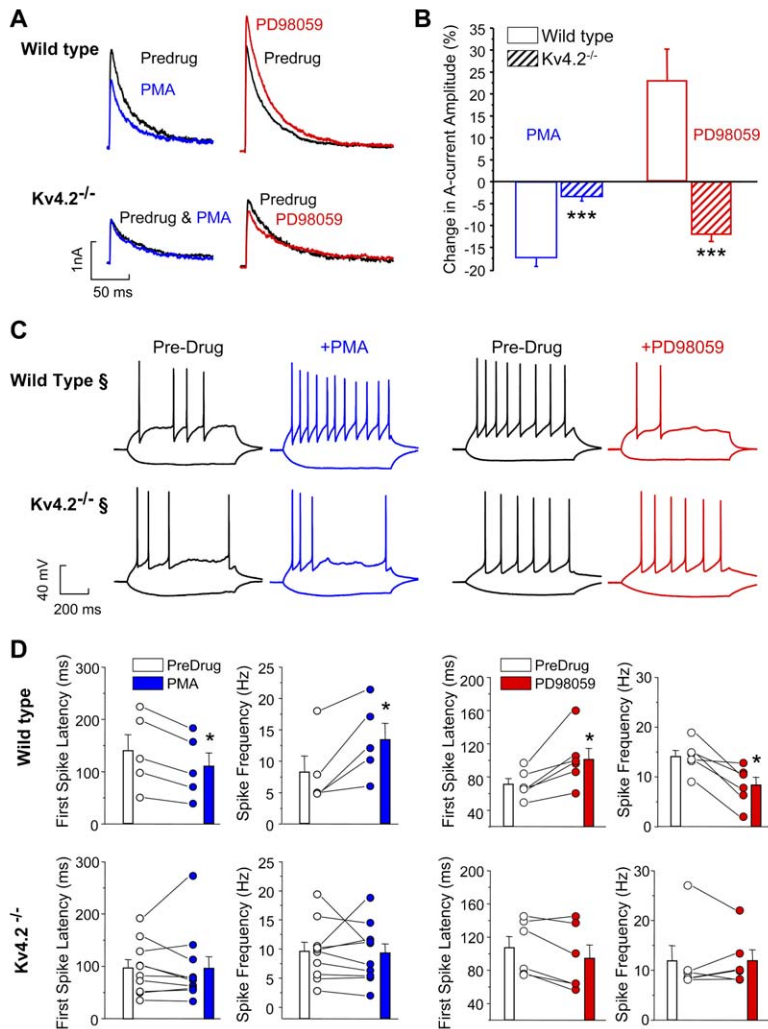

Figure 6. Kv4.2 Is Required for ERK-Dependent Modulation of A-Type K+ Currents and Neuronal Excitability in Spinal Cord Dorsal Horn Neurons

(A) Representative A-type currents recorded before (pre-drug, black traces) and after application of PMA (5 μM, blue traces) or PD98059 (20 μM, red traces) to wt or $Kv4.2^{-/-}$ neurons.

(B) Grouped data showing the effect of PMA and PD98059 on peak amplitude of A-type currents in wt (open bars) and $Kv4.2^{-/-}$ (hatched bars) neurons. Values represent mean ± SEM, n = 6–9 neurons. ***$p < 0.001$, t test comparing genotypes.

(C) Representative action potentials recorded in wt and $Kv4.2^{-/-}$ dorsal horn neurons (lamina I-II) of spinal cord slices before (pre-drug) and after application of PMA (5 μM) or PD98059 (20 μM).

(D) Effect of PMA (blue) and PD98059 (red) on first-spike latency and spike frequency in wt and $Kv4.2^{-/-}$ neurons. Circles show individual values measured before (open circles) or after (closed circles) drug application. Bars represent mean ± SEM of all cells; n = 5–10. *$p < 0.05$, paired Students' t test.

§ For experiments in panels (C) and (D), current injection amplitudes were adjusted to elicit roughly equivalent firing patterns in the wt and $Kv4.2^{-/-}$ neurons in the pre-drug conditions to allow direct comparison of the drug effects in the different genotypes. Thus, the baseline firing properties are not representative of differences between the genotypes when equivalent currents are injected, as shown in Figure 4.

Figure 5 and Figure 7B to the FVB mice in Figure 7E and Figure S1). Mice used in Figures 7C and 7D were ICR wild-type mice.

The carrageenan model of inflammatory pain in rats and mice is also associated with increased ERK activation in the spinal dorsal horn (Galan et al., 2002). In the carrageenan model, we found a phenotype in the $Kv4.2^{-/-}$ mice that is very similar to what we observed in the formalin model. Thus, while $Kv4.2^{-/-}$ mice were hypersensitive to touch relative to $Kv4.2^{+/+}$ mice prior to carrageenan injection in the hindpaw, following inflammation the $Kv4.2^{+/+}$ mice developed hypersensitivity to touch in both the ipsilateral and contralateral paw, whereas the mechanical-withdrawal thresholds in $Kv4.2^{-/-}$ mice did not differ from baseline for the ipsilateral or contralateral paw (Figure 7E).

In addition to the above-mentioned models of inflammatory pain, the CCI model of neuropathic pain has been shown to be associated with increased spinal ERK activity, and intrathecally administered MEK inhibitors reduce allodynia in this model at concentrations that are effective at inhibiting ERK activity (Ciruela et al., 2003). $Kv4.2^{-/-}$ mice and $Kv4.2^{+/+}$ littermates both developed mechanical hypersensitivity following CCI surgery (see Figure S1 in the Supplemental Data). Interestingly, $Kv4.2^{-/-}$ and $Kv4.2^{+/+}$ mice reached identical absolute withdrawal thresholds following nerve injury. However, withdrawal thresholds in $Kv4.2^{+/+}$ mice remained significantly reduced relative to pre-CCI baselines for >35 days after surgery, whereas in $Kv4.2^{-/-}$ mice, withdrawal thresholds returned to prebaseline measurements by 28 days post-CCI. Whether this represents a physiologically significant effect is not clear. In total, our behavioral data point to a more critical role for ERK modulation of Kv4.2 in inflammatory pain.

Phosphorylation Site S616 Mediates ERK Modulation of Kv4.2

Our working model is that Kv4.2 is directly phosphorylated by ERK, and this mediates a component of central sensitization underlying inflammatory pain. As mentioned above, previous studies using purified fusion proteins identified three sites that were phosphorylated on the Kv4.2 intracellular domains by ERK in vitro (Adams et al., 2000). However, to date there are no data suggesting which, if any, of these sites are important for ERK-dependent modulation of Kv4.2-mediated currents in neurons, and it is formally possible that this modulation is dependent on ERK phosphorylation of an accessory protein rather than Kv4.2 itself. To begin to address

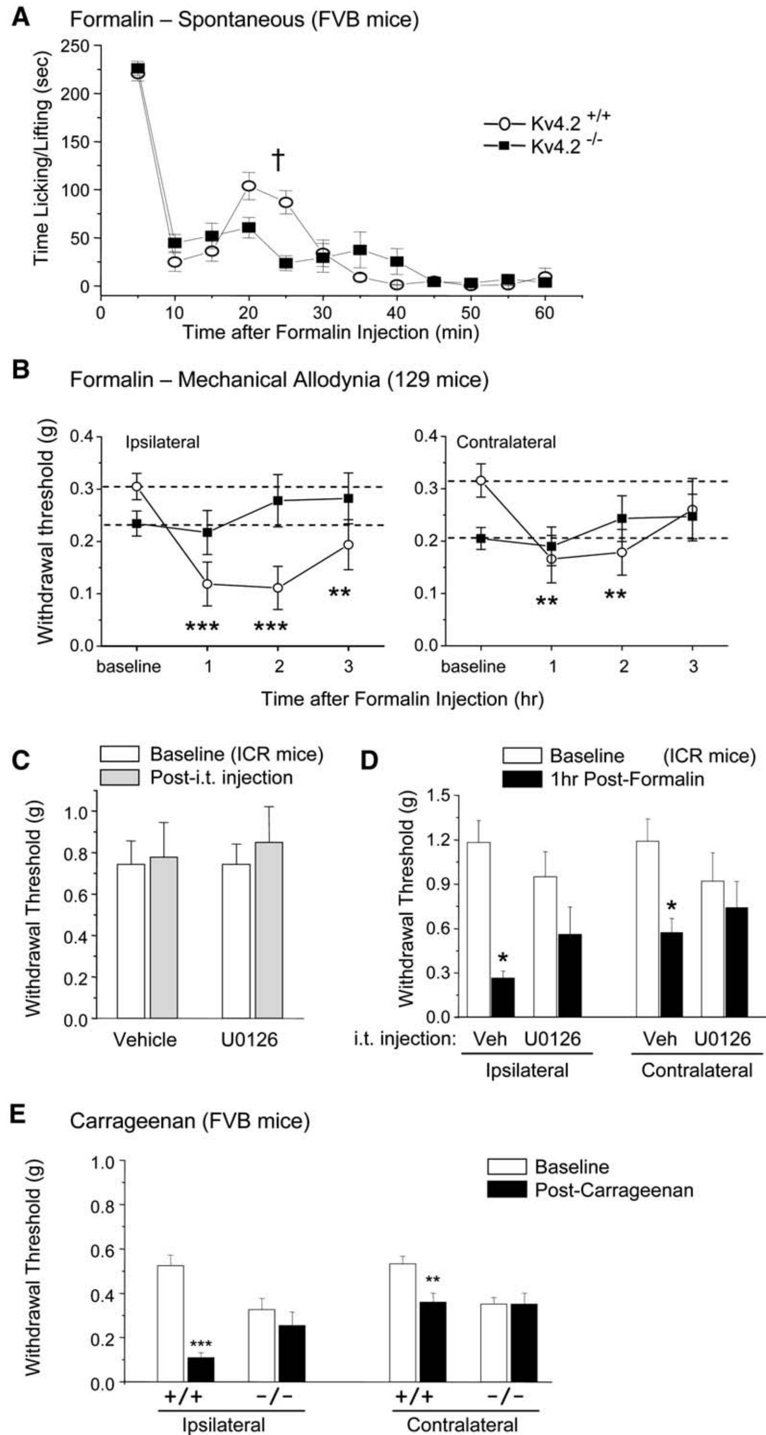

Figure 7. Reduced ERK-Dependent Pain Plasticity in *Kv4.2*$^{-/-}$ Mice

(A) Formalin induced spontaneous nociceptive behavior in FVB mice (n = 16–18).

(B) Time course of formalin-induced mechanical allodynia (n = 8–18) in the injected (ipsilateral) paw or the noninjected (contralateral) paw relative to baselines taken before injection of formalin (dashed line) in 129 mice.

(C) Effect in wt ICR mice of i.t. injection of U0126 (2 nmol) on basal mechanical thresholds (n = 10 each).

(D) Effect in wt ICR mice of i.t. injection of U0126 (2 nmol) on formalin-induced mechanical allodynia measured 1 hr after formalin injection (n = 9–10). *p < 0.05.

(E) Carrageenan-induced mechanical hypersensitivity in *Kv4.2*$^{-/-}$ mice and wild-type littermates (FVB mice, n = 5–6).

For all figures, all values represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Closed symbols and bars represent *Kv4.2*$^{-/-}$ results, open symbols and bars represent wild-type littermates. †p < 0.05, ANOVA, comparing *Kv4.2*$^{+/+}$ and *Kv4.2*$^{-/-}$ from 10–30 min, 35–45 min, and 50–60 min time periods are not significantly different. ANOVA for total formalin second phase (10–60 min) was not significant.

this issue, we generated point mutations in each of the three biochemically identified ERK phosphorylation sites on Kv4.2 and used these to determine which of the sites (if any) mediates ERK-dependent modulation of Kv4.2. We transfected dorsal horn neurons from Kv4.2 knockout mice with either wild-type Kv4.2 or alanine mutants of the ERK phosphorylation sites T602, T607, or S616 (Adams et al., 2000). Transfection of *Kv4.2*$^{-/-}$ dorsal horn neurons with wild-type Kv4.2 restored A-type currents, and these currents were inhibited via an ERK-dependent mechanism by PMA, similar to what we observed in wild-type neurons (Figures 8A and 8B and see Figures 6A and 6B). A-type currents were also restored in *Kv4.2*$^{-/-}$ dorsal horn neurons transfected with the T602A, T607A, and S616A ERK phosphorylation site mutants, and biophysical properties of these currents were indistinguishable from those mediated by wild-type Kv4.2 (Figures 8C and 8D). In neurons transfected with Kv4.2(T602A) or Kv4.2(T607A), ERK-dependent inhibition of A-type currents was similar

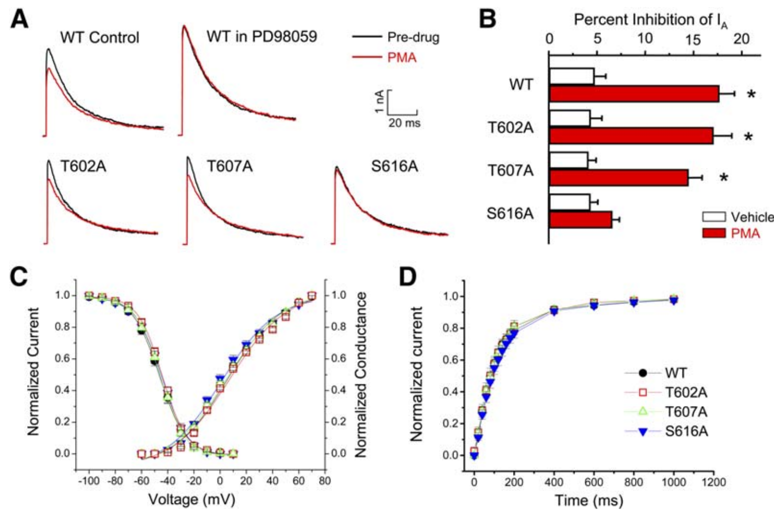

Figure 8. The S616 Phosphorylation Site Mediates ERK Modulation of Kv4.2-Mediated A-Type Currents in Spinal Cord Dorsal Horn Neurons

(A) Representative A-type currents recorded from *Kv4.2*$^{-/-}$ neurons transfected with EGFP + wild-type Kv4.2 or EGFP + Kv4.2 phosphorylation site mutants (T602A, T606A, or S616A) before (pre-drug, black traces) and after application of PMA (5 μM, red traces). The MEK inhibitor PD98059 (20 μM) blocks PMA-induced modulation of A-type currents transfected with wild-type Kv4.2, as well as those transfected with T602A or T607A (not shown).

(B) Effect of PMA on peak amplitude of A-type currents in *Kv4.2*$^{-/-}$ neurons transfected with EGFP + wild-type Kv4.2 or + Kv4.2 phosphorylation site mutants. Values represent mean ± SEM, n = 6–9 neurons. ***$p < 0.001$, compared with control (vehicle).

(C) Steady-state inactivation and activation curves from *Kv4.2*$^{-/-}$ neurons transfected with EGFP + wild-type Kv4.2 or + Kv4.2 phosphorylation site mutants (n = 8–14).

(D) Recovery from inactivation of A-type currents in *Kv4.2*$^{-/-}$ neurons transfected with EGFP + wild-type Kv4.2 or + Kv4.2 phosphorylation site mutants (n = 8–9).

to that observed in neurons transfected with wild-type Kv4.2. In contrast, ERK-dependent modulation of A-type currents was absent in dorsal horn neurons transfected with Kv4.2(S616A) (Figure 8).

Discussion

In the present study, we identify the Kv4.2 subunit as a prominent contributor to A-type currents in dorsal horn neurons and demonstrate that this subunit is a critical site of regulation in pain processing. Kv4.2 knockout mice have reduced A-type currents in dorsal horn neurons, and this leads to increased excitability of these neurons associated with a slight membrane depolarization. These lamina I-II neurons receive synaptic input from primary afferent nociceptors, and one would hypothesize that because of these alterations to neuronal excitability, a small synaptic input would elicit more action potentials in dorsal horn neurons from *Kv4.2*$^{-/-}$ mice than in those from wild-type mice. Accordingly, we observed an increased sensitivity to touch and heat in *Kv4.2*$^{-/-}$ mice relative to their *Kv4.2*$^{+/+}$ littermates. We also show that ERK-dependent modulation of neuronal excitability found in wild-type dorsal horn neurons is completely absent in dorsal horn neurons from *Kv4.2*$^{-/-}$ mice. Consistent with this absence of ERK-dependent modulation of neuronal excitability, we find that *Kv4.2*$^{-/-}$ mice have significant deficits in several pain behavioral models in which ERK signaling has been implicated. Taken together, our results suggest that Kv4.2-containing K+ channels represent a critical node of modulation that regulates transmission of nociceptive signals from the periphery to the brain.

Because Kv4.2 is a known substrate for ERK phosphorylation (Adams et al., 2000), it is reasonable to hypothesize that the reduction in ERK-dependent behavioral sensitization in Kv4.2 knockout mice is due to the requirement for ERK-mediated phosphorylation and inhibition of Kv4.2-containing K+ channels, which normally leads to enhanced excitability of superficial dorsal horn neurons (Hu and Gereau, 2003; Hu et al., 2003; Karim et al., 2006). The results of our analysis of ERK phosphorylation site mutants of Kv4.2 are consistent with this hypothesis and suggest that S616 is the functionally relevant phosphorylation site on Kv4.2. A direct test of this model would be a genetically modified mouse that carries a mutation in the S616 ERK phosphorylation site of Kv4.2. The prediction would be that these animals would have relatively normal A-type currents in dorsal horn neurons but no modulation of this current by ERK activity. At the behavioral level, the prediction would be that these mice would have normal baseline pain sensitivity but have reduced hypersensitivity in inflammatory pain models. This would be slightly different than what we observe in the *Kv4.2*$^{-/-}$ animals, which have reduced A-type currents and resultant hyperexcitability of dorsal horn neurons, leading to nociceptive hypersensitivity under baseline conditions. In contrast, the ERK-dependent behavioral hypersensitivity that occurs following inflammation should be absent both in the *Kv4.2*$^{-/-}$ mice and in the Kv4.2 ERK phosphorylation site mutant mice.

Although the model described above can account for the behavioral differences observed in *Kv4.2*$^{-/-}$ mice relative to wild-type mice, it is formally possible that because the *Kv4.2*$^{-/-}$ animals are hypersensitive under baseline conditions, they have simply reached their maximum level of sensitivity. For several reasons, we believe that this is not the case. First, the withdrawal thresholds for wild-type mice after formalin- or carrageenan-induced inflammation are significantly lower than the *Kv4.2*$^{-/-}$ mice in the basal state and after

inflammation (Figures 7B and 7E). Second, the $Kv4.2^{-/-}$ mice are clearly able to be made more hypersensitive to touch, as there is significant hypersensitivity in the $Kv4.2^{-/-}$ mice following CCI relative to presurgery baselines (Figure S1). These data are consistent with the hypothesis that the lack of hypersensitivity in the $Kv4.2^{-/-}$ mice relative to $Kv4.2^{+/+}$ littermates is due to reduced plasticity in the $Kv4.2^{-/-}$ mice and not due to a behavioral "floor effect."

The finding that $Kv4.2^{-/-}$ mice still have some hypersensitivity in the CCI model of persistent neuropathic pain is interesting given the diversity of actions of ERK signaling in neurons (Ji et al., 2003). It is possible that ERK phosphorylation and modulation of Kv4.2-containing K+ channels mediates a component of central sensitization but that transcription-dependent changes underlie other long-term components of ERK-dependent central sensitization that are not reduced in the Kv4.2 knockouts (Ji and Rupp, 1997; Ji et al., 2002; Woolf and Costigan, 1999). Our data are consistent with the hypothesis that Kv4.2 modulation plays a more significant role in inflammatory pain than in longer-term neuropathic pain conditions, where ERK-dependent transcriptional regulation may be more important. The $Kv4.2^{-/-}$ animals and potential phosphorylation site mutant animals may provide useful tools for future studies examining the relative importance of acute phosphorylation and modulation of K+ channels and transcription/translation-dependent changes in mediating various forms of long-term pain hypersensitivity.

Although our data are consistent with a critical role of ERK modulation of Kv4.2-containing potassium channels in superficial spinal dorsal horn neurons in mediating behavioral sensitization following inflammation, it is certainly true that Kv4.2 is expressed in many areas of the nervous system. Thus, it is possible that Kv4.2 plays important roles in other parts of the pain neuraxis and a component of the alterations in behavior observed in the Kv4.2 knockouts could be mediated by altered physiology in these areas in addition to the spinal cord. We were particularly curious to see whether Kv4.2 might be impacting the physiology of nociceptive primary afferent neurons, but we were unable to detect any Kv4.2 expression in the mouse dorsal root ganglion by immunostaining (data not shown). In addition, the superficial laminae of the spinal cord contain both excitatory and inhibitory neurons, and excitatory neurons can be projection neurons or interneurons. Because we do not know the transmitter phenotype of all of the neurons from which we have recorded, the specific cellular circuitry impacted by these changes remains to be determined.

Our results reveal a link between Kv4.2 and ERK signaling in the spinal cord and support a model in which Kv4.2-containing potassium channels in spinal superficial dorsal horn neurons are modulated by ERKs to induce a component of central sensitization. Our data further implicate direct phosphorylation of Kv4.2 at S616 in this process. ERK-dependent phosphorylation of Kv4.2 and modulation of A-type K+ currents appears to underlie plasticity in hippocampal pyramidal neurons associated with long-term potentiation (LTP) (Kim et al., 2005; Schrader et al., 2005; Watanabe et al., 2002). There has been considerable interest in the parallels between LTP and central sensitization (Basbaum, 1996; Ji et al., 2003; Karim et al., 2001; Sandkuhler and Liu, 1998; Willis, 2002); the present study supports the idea that LTP and central sensitization may share common mechanisms, as our studies and previous work have demonstrated the importance of ERK signaling and Kv4.2 in both of these processes (Ji et al., 2003). Whether these similarities translate to similarities in the forms of synaptic plasticity observed in dorsal horn neurons to mediate central sensitization is worthy of extensive study.

The results presented here advance our understanding of the mechanisms underlying pain processing and are especially relevant to our understanding of inflammation-induced pain hypersensitivity. Our findings suggest that manipulation of the ERK-Kv4.2 signaling pathway could be useful for novel therapies for the treatment of pain.

Experimental Procedures

Cell Culture and Spinal Cord Preparation

Primary cultures of spinal cord superficial dorsal horn neurons were prepared from 4- to 8-day-old CD1 mice and 129SvEv wild-type or $Kv4.2^{-/-}$ mice as previously described (Hugel and Schlichter, 2000). Lumbar spinal cord slices (300–350 μm) were prepared from 6- to 10-day-old CD1 or 129SvEv mice as previously described (Edwards et al., 1989) and maintained in artificial cerebrospinal fluid (ACSF) containing (in mM) 118 NaCl, 3 KCl, 24 $NaHCO_3$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 1 $CaCl_2$, and 12 glucose at room temperature under continuous oxygenation for 1–4 hr.

Transfection of Dorsal Horn Neurons

The EGFP-Kv4.2dn construct was generously provided by Dr. Paul Pfaffinger (Baylor College of Medicine). Plasmid DNA was isolated using the Qiagen plasmid maxi protocol. Spinal cord dorsal horn neurons were cultured for 24 hr and then transfected with plasmid DNA constructs (0.45–0.9 μg/well) overnight using LipofectAMINE Plus or 2000 reagent according to the manufacturer's protocol (Invitrogen Carlsbad, CA). Patch-clamp recordings were performed beginning $\sim$16 hr following transfection. Point mutations in ERK phosphorylation sites were generated as described previously (Gereau and Heinemann, 1998).

Electrophysiological Recording

Whole-cell recordings were performed using standard procedures at room temperature using either an AXOPATCH 200B amplifier and CLAMPEX 8.0 software (Axon Instruments, Union City, CA) or an EPC-10 amplifier and Pulse v8.62 software (HEKA Elektronik, Lambrecht, Germany) as previously described (Hu and Gereau, 2003; Hu et al., 2003). Electrode resistances were 3–6 MΩ with series resistances around 6–15 MΩ and were compensated by $\geq$60%. For voltage-clamp recordings in cultured neurons, the bath solution was Hank's solution (HBSS) (in mM: 137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 4.2 $NaHCO_3$, 0.3 Na_2HPO_4, 5.6 glucose) containing 500 nM TTX and 2 mM $CoCl_2$ to block voltage-gated Na^+ currents, Ca^{2+} currents, and Ca^{2+}-activated K+ currents. The electrode solution contained (in mM) 140 KCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, 3 Na_2ATP, and 0.3 Na_2GTP (pH 7.4). The membrane voltage was held at -80 mV, and transient potassium currents (I_A) were isolated by a two-step voltage protocol as previously described (Hu et al., 2003). To determine the voltage-dependent activation, voltage steps of 500 ms were applied at 5 s intervals in +10 mV increments from -70 mV to a maximum of +70 mV. To determine the voltage-dependent inactivation, conditioning prepulses ranging from –120 mV to +40 mV were applied at 5 s intervals in +10 mV increments for 150 ms followed by a step to +40 mV for 500 ms. To determine time-dependent recovery from inactivation, conditioning pulses (40 mV) were applied for 500 ms followed by steps to +40 mV for 500 ms in 20 or 200 ms incremental duration. For current-clamp recording in slice, the bath solution was ACSF bubbled with 95% O_2-5% CO_2. Intracellular solution contained

(in mM) 140 $KMeSO_4$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 3 Na_2ATP, and 0.3 Na_2GTP (pH 7.4). Action potentials were generated by current injection from a holding potential of −70 mV for rheobase or −77 mV for drug application.

Generation of Kv4.2 Knockout Mice

Kv4.2-deficient mice were generated as we have previously described (Guo et al., 2005). Briefly, the targeting vector was constructed in the pPNT vector using 129Sv genomic DNA from a phage isolate. For the 3′ arm, a 7.1 kb AflII genomic fragment, downstream of the first coding exon, was inserted into a SrfI site that previously been introduced between the KpnI and EcoRI sites of the vector. For the 5′ arm, a 3.1 kb SpeI-ScaI genomic fragment upstream of the predicted start codon was inserted between the NotI and the XhoI sites. Thus, the coding region of the first exon of Kv4.2 was replaced with the *neor* gene. ES cells (gift of Dr. Andras Nagy, University of Toronto) were electroporated with the targeting vector and selected with G418 and gancyclovir. Clones were injected into blastocysts to generate chimeric mice, which were crossed to 129/SvEv mice to produce *Kv4.2*$^{+/-}$ mice. Wild-type and *Kv4.2*$^{-/-}$ littermate mice generated from heterozygous intercrosses were used for behavior tests.

Western Blot Analysis

The lumbar section of the spinal cord was dissected from adult 129SvEv wild-type or *Kv4.2*$^{-/-}$ mice and homogenized using homogenization buffer (HB) (Tris 20 mM, pH 7.5, EDTA 1 mM, $Na_4P_2O_7$ 1 mM, aprotinin 25 μg/ml, leupeptin 25 μg/ml, Na_3VO_4 0.2 mg/ml, and PMSF 0.4 mM). Membrane proteins were separated and electrophoresed in 10% SDS polyacrylamide gels. The blots were blocked with 5% milk and probed with anti-Kv4.2 primary antibody (1:500, Chemicon). The blots were incubated with HRP-conjugated secondary antibody (1:20,000, Cell Signaling) and developed with the SuperSignal West Femto reagent (Pierce).

Immunohistochemistry

Thirty-micron sections of paraformaldehyde-fixed lumbar spinal cord from adult 129SvEv wild-type or *Kv4.2*$^{-/-}$ mice were stained with rabbit anti-Kv4.2 (1:800, Alomone) or rabbit anti-Kv4.3 primary antibody (1:200, Chemicon). Sections were then incubated in biotinylated secondary antibody (1:200, Vector) and treated in extrAvidin peroxidase (1:1000, Sigma). Detection was performed using a diaminobenzidine (DAB) substrate kit (Vector).

Behavioral Studies

All behavioral tests were performed blind using 7- to 9-week-old mice. All the experiments were done in accordance with the guidelines of the National Institutes of Health and The International Association for the study of Pain and were approved by the Animal Care and Use Committee of Washington University School of Medicine. Wild-type and *Kv4.2*$^{-/-}$ littermate mice of both sexes were tested except where mentioned.

Motor Function

Mice were tested for motor function using the accelerating rotarod (4–40 rpm) (UGO Basile, Varese, Italy). The time spent on the rotarod was recorded.

Basal Sensitivity to Mechanical and Thermal Stimulation

Mechanical sensitivity was measured in male mice using von Frey filaments (North Coast Medical, Inc., San Jose, CA) as previously described (Yang and Gereau, 2003). The smallest monofilament that evoked paw-withdrawal responses on three out of five trials was taken as the mechanical threshold. The tail pressure test was conducted using the Basile Analgesy-Meter, and the response (struggle/vocalization) threshold was measured as previously described (Nassar et al., 2004). Hot plate latencies were measured as the time taken for a mouse to lick or shake its hindpaw at hot plate temperatures of either 52ºC or 56ºC.

Formalin Model

The formalin test was performed by injection of 5% formalin subcutaneously into the plantar surface of the right hindpaw. The total time spent in spontaneous nociceptive behavior (licking and lifting of the injected paw) was recorded in 5 min intervals for 1 hr as previously described (Karim et al., 2001). Mechanical allodynia was measured in male mice from 1–3 hr after injection of formalin using von Frey filaments. For experiments analyzing the effect of U0126 on formalin-induced allodynia, mechanical thresholds were measured 1 hr after formalin injection. Biochemical studies were performed to test the optimal dose and timing for U0126 intrathecal injections and revealed that intrathecal injection of 2 nmol of U0126 was able to inhibit ERK activation for up to 2 hr after intrathecal injection (data not shown). Based on this finding, U0126 was injected 15 min prior to formalin injection, and allodynia was measured 1 hr after formalin injection.

Carrageenan Model

Two percent carrageenan was injected subcutaneously into the plantar surface of the right hindpaw. Mechanical hypersensitivity was measured in male mice 2 hr after injection of carrageenan, using von Frey filaments as described above.

Chronic Constrictive Injury Model

CCI of the sciatic nerve was induced as previously described (Bennett and Xie, 1988) in male FVB mice. Briefly, under pentobarbital sodium anesthesia, the left sciatic nerve was exposed at mid-thigh, and two ligatures were loosely ligated proximal to the sciatic's trifurcation at ∼1.0 mm intervals with 6-0 chromic gut sutures. Mechanical hypersensitivity was measured on 1, 4, 7, 10, 14, 21, 28, and 35 days post-CCI, using von Frey filaments as described above.

Drug Application

Stock solutions of phorbol 12-myristate 13-acetate (PMA), PD 098059 (Sigma-Aldrich, St. Louis, MO), and U-0126 (Calbiochem, La Jolla, CA) were made in DMSO and diluted to final concentrations in HBSS for bath applications or in PBS for intrathecal injections.

Data Analysis

Offline evaluation was done using clampfit 8.0 (Axon Instrument) or Pulse v8.62 (HEKA) and Origin (Microcal Software Inc., Northampton, MA). Data are expressed as original traces and/or as mean ± SEM. The voltage dependence of activation and inactivation of the I_A was fitted with the Boltzmann function as previously described (Hu et al., 2003). Behavioral experiments were statistically analyzed by ANOVA (or repeated-measures ANOVA when appropriate as indicated) followed by the appropriate post hoc tests. Paired or two-sample Student's t test was used when comparisons were restricted to two means. Error probabilities of $p < 0.05$ were considered statistically significant.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/50/1/89/DC1/.

Acknowledgments

The authors wish to thank P. Pfaffinger for generously providing the Kv4.2dn construct; and C. Qiu, S. Shalin, and B. McGill for help with mouse colony maintenance, immunostaining, and dorsal horn neuronal transfections, respectively. This work was supported by grants from the NIH (R.W.G., Y.C., J.M.N., and T.L.S.), Arthritis Foundation (F.K.), and the Paralyzed Veterans of America Spinal Cord Research Foundation (R.W.G.).

Received: July 8, 2005
Revised: August 10, 2005
Accepted: March 3, 2006
Published: April 5, 2006

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