Behavioral/Systems/Cognitive

Spinal G-Protein-Gated K$^+$ Channels Formed by GIRK1 and GIRK2 Subunits Modulate Thermal Nociception and Contribute to Morphine Analgesia

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G-protein-gated potassium (K$^+$) channels are found throughout the CNS in which they contribute to the inhibitory effects of neurotransmitters and drugs of abuse. Recent studies have implicated G-protein-gated K$^+$ channels in thermal nociception and the analgesic action of morphine and other agents. Because nociception is subject to complex spinal and supraspinal modulation, however, the relevant locations of G-protein-gated K$^+$ channels are unknown. In this study, we sought to clarify the expression pattern and subunit composition of G-protein-gated K$^+$ channels in the spinal cord and to assess directly their contribution to thermal nociception and morphine analgesia. We detected GIRK1 (G-protein-gated inwardly rectifying K$^+$ channel subunit 1) and GIRK2 subunits, but not GIRK3, in the superficial layers of the dorsal horn. Lack of either GIRK1 or GIRK2 was correlated with significantly lower expression of the other, suggesting that a functional and physical interaction occurs between these two subunits. Consistent with these findings, GIRK1 knock-out and GIRK2 knock-out mice exhibited hyperalgesia in the tail-flick test of thermal nociception. Furthermore, GIRK1 knock-out and GIRK2 knock-out mice displayed decreased analgesic responses after the spinal administration of higher morphine doses, whereas responses to lower morphine doses were preserved. Qualitatively similar data were obtained with wild-type mice after administration of the G-protein-gated K$^+$ channel blocker tertiapin. We conclude that spinal G-protein-gated K$^+$ channels consisting primarily of GIRK1/GIRK2 complexes modulate thermal nociception and mediate a significant component of the analgesia evoked by intrathecal administration of high morphine doses.

Key words: knock-out; opioid; tail-flick; Kir3; potassium; pain

Introduction

Nociception involves multiple spinal and supraspinal circuits and neurotransmitter systems (Millan, 1999, 2002). Primary afferent fibers transmit nociceptive information from the periphery to secondary dorsal horn neurons. This relay is subject to modulation by spinal interneurons and supraspinal projections originating in the periaqueductal gray (PAG) and rostroventral medulla. Opiate analgesics such as morphine bind preferentially to μ-opioid receptors (MORs) and trigger antinociception by inhibiting neuronal targets, including supraspinal GABAergic interneurons, primary afferent neurons, and secondary dorsal horn neurons (Yaksh et al., 1988; Yaksh, 1997).

G-protein-gated, inwardly-rectifying K$^+$ (GIRK) channels contribute to the postsynaptic inhibition triggered by many neurotransmitters, including opioids (North, 1989). GIRK channels

are complexes consisting of GIRK/Kir3 subunits (Dascal, 1997; Mark and Herlitze, 2000; Sadja et al., 2003). Four GIRK subunits (GIRK1–GIRK4) have been identified and can form homotetrameric and heterotetrameric channels gated by the G$\beta\gamma$ subunit of G$_{i/o}$ G-proteins (Dascal, 1997; Mark and Herlitze, 2000; Sadja et al., 2003). Atrial GIRK1/GIRK4 channels mediate the heart rate decrease caused by parasympathetic discharge (Wickman et al., 1998), whereas channels formed by various combinations of GIRK1, GIRK2, and GIRK3 mediate inhibition in the nervous system (Lesage et al., 1994; Karschin et al., 1996; Luscher et al., 1997; Torrecilla et al., 2002). Although the relevance of subunit composition is not fully appreciated, channels formed by different GIRK subunit combinations exhibit distinct single-channel profiles, whole-cell current kinetics, and G$\beta\gamma$ sensitivity (Duprat et al., 1995; Krapivinsky et al., 1995; Lesage et al., 1995; Jelacic et al., 2000). It should also be noted that GIRK1 homomultimeric complexes are not functional (Hedin et al., 1996; Kennedy et al., 1996).

Studies involving knock-out mice have offered insight into GIRK channel subunit composition *in vivo* and have implicated these channels in postsynaptic inhibition and various behaviors. For example, hippocampal pyramidal neurons and cerebellar granule cells from GIRK2 knock-out mice exhibited a loss of postsynaptic inhibition evoked by GABA (Luscher et al., 1997;

Slesinger et al., 1997). Decreased opioid-induced currents were observed in locus ceruleus neurons from GIRK2 knock-out mice, with opioid inhibition virtually absent in neurons from GIRK2/GIRK3 knock-out mice (Torrecilla et al., 2002). GIRK2 knock-out mice display spontaneous seizures, hyperactivity, reduced cocaine self-administration behavior, and altered responses to ethanol (Signorini et al., 1997; Blednov et al., 2001a,b; Morgan et al., 2003). In addition, GIRK2-containing channels have been implicated in thermal and chemical nociception, gender differences observed in mice in tests of nociception, and the analgesic effects of morphine, ethanol, oxotremorine, baclofen, clonidine, nicotine, and WIN 55,212-2 (Ikeda et al., 2000; Marker et al., 2002; Blednov et al., 2003; Mitrovic et al., 2003).

Previously, we identified roles for GIRK2- and GIRK3-containing channels in the modulation of thermal nociceptive thresholds and in the analgesic effect of morphine (Marker et al., 2002). Because this previous and related studies involved systemic morphine administration, the location of GIRK channels relevant to thermal nociception and opioid antinociception remains unclear. Here, we tested the hypothesis that spinal GIRK channels modulate thermal nociception and mediate morphine analgesia.

Materials and Methods

Behavioral subjects. All animal use was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Efforts were made to minimize the pain and discomfort of the animals throughout the study. Mice were housed in a temperature-controlled room on a 12 hr light/dark cycle, with food and water available *ad libitum*. The generation of GIRK1 knock-out (Bettahi et al., 2002), GIRK2 knock-out (Signorini et al., 1997), GIRK3 knock-out (Torrecilla et al., 2002), and GIRK2/GIRK3 double knock-out (Torrecilla et al., 2002) mice was described previously. The GIRK null mutations were backcrossed through at least nine rounds against the C57BL/6 mouse strain before initiating this study. Genotypes were determined by PCR of crude DNA samples from tail biopsies (Wickman et al., 1998). Both male and female mice were tested in the tail-flick test between 6 and 7 weeks of age. Separate cohorts of mice were used for each drug condition, and the experimenter was blind to subject genotype during behavioral testing.

Immunohistochemistry. Adult female wild-type and GIRK knock-out mice were anesthetized and subjected to transcardial perfusion with Ca^{2+}-free Tyrode's solution, followed by a 4% paraformaldehyde-based fixative, and finally with 5% sucrose solution. Spinal cords were then removed and postfixed at room temperature for 30 min, followed by incubation in 5% sucrose overnight at 4°C. Tissue was sectioned by cryostat at a thickness of 10 μm, and sections were incubated with primary antibody overnight at 4°C. Anti-GIRK1 (Sigma, St. Louis, MO) and anti-GIRK2 (Alomone Labs, Jerusalem, Israel) antibodies were diluted in PBS containing 0.1% Triton X-100 and applied to sections at dilutions of 0.5 μg/ml and 1:200, respectively. The following morning, sections were washed with PBS (three times for 20 min each) and incubated for 2 hr at room temperature with a 1:500 dilution of a Cy3-conjugated, donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) for immunofluorescence detection. Sections were washed with PBS (three times for 20 min each), dehydrated through an increasing gradient of ethanol concentrations, cleared with xylenes, and coverslipped using DPX (Fluka, Ronkonkoma, NY). Images were collected using a Nikon (Melville, NY) TE200 microscope and a DVC-1412M camera (Digital Video Company, Austin, TX).

Drugs. Morphine sulfate (Sigma) and rTertiapin-Q (Alomone Labs) were suspended in 0.9% saline solutions. Drugs were administered intrathecally by direct lumbar puncture as described previously (Hylden and Wilcox, 1980). The highest doses of intrathecal morphine (3 nmol) and tertiapin (100 pmol) administered were chosen on the basis of responses observed in pilot studies, in which morphine doses >3 nmol induced writhing and biting of the flank, and tertiapin doses >100 pmol led

consistently to prolonged episodes of uncontrollable twitching and spasms of the lower torso.

Tail-flick test. Thermal nociception was assessed using the warm-water immersion tail-flick assay. On 2 d before testing, as well as on the day of testing, mice were acclimated to the testing environment for ≥1 hr. During tail-flick testing, mice were secured firmly with the full tail exposed using a soft cloth. Tails were immersed in a sequential manner in water baths set at 49°C, 52.5°C, and 55°C and were wiped dry between dips. The mice were then injected intrathecally with saline or drug(s), and tail-flick latencies were measured again after 10 min. To prevent tissue damage, cutoff values were set at 20, 12, and 6 sec for the 49°C, 52.5°C, and 55°C bath exposures. The investigator was blind to drug status throughout the experiment. Water bath temperatures were measured daily using a Traceable RTD platinum thermometer (Control Company, Friendswood, TX) and adjusted as necessary to ensure consistency throughout the study.

Data analysis. Data are presented throughout as the mean ± SEM. For experiments involving the injection of morphine (see Fig. 3) or tertiapin alone (see Fig. 4), drug effect is presented as the difference between preinjection and postinjection tail-flick latencies (ΔTF latency). For experiments involving the coinjection of morphine and tertiapin (see Fig. 5), ΔTF latency values for the saline- and tertiapin-treated mice were normalized to offset the acute hyperalgesic effect of tertiapin, thereby permitting the direct comparison of morphine effects between the two groups of mice. The normalization is described by the following equation, where ΔTF (average) is the average ΔTF latency determined for the control and tertiapin groups after the injection of saline and 30 pmol of tertiapin, respectively: $n\Delta$TF latency = [(drug latency − baseline latency) − ΔTF (average)]. Baseline, ΔTF latency, and $n\Delta$TF latency measures were analyzed at each temperature by ANOVA to determine whether subject gender, genotype, and drug status were significant predictors of behavioral performance. A significant effect of gender on baseline tail-flick latency was observed; as such, data from male and female wild-type and knock-out mice were considered separately. In contrast, no effect of gender was observed for ΔTF latency and $n\Delta$TF latency measures; as such, data from male and female mice were combined. All *post hoc* analysis was performed using Tukey's honestly significant difference test. Differences were considered to be significant if $p < 0.05$.

Results

Previously, we demonstrated that GIRK1 and GIRK2, and to a lesser extent GIRK3, were found in membrane protein extracts isolated from the mouse spinal cord (Marker et al., 2002). To determine the precise distribution of GIRK subunits within the spinal cord, we immunostained spinal cord sections taken from the lumbar enlargement of adult wild-type and GIRK knock-out mice. Both GIRK1 and GIRK2 subunits, but not GIRK3, were detected in the spinal cord gray matter in wild-type sections, with the most intense labeling observed in the superficial layers of the dorsal horn (Fig. 1A,B). Throughout the gray matter, GIRK2 staining was characterized by thread-like and punctate structures; comparable structures were not evident in sections stained with GIRK1 antibody. Importantly, the specificities of the GIRK1 and GIRK2 antisera were confirmed by the absence of signal in sections from the appropriate GIRK knock-out animal (Fig. 1C,F).

Because constitutive gene ablation can promote compensatory changes in the expression of related proteins, we examined residual GIRK subunit expression in tissue from GIRK1, GIRK2, and GIRK3 knock-out mice. We observed dramatically lower levels of GIRK1 in GIRK2 knock-out tissue (Fig. 1E) and lower levels of GIRK2 in GIRK1 knock-out tissue (Fig. 1D). Although we failed to detect GIRK3 expression in wild-type spinal cord sections, we did observe slightly lower levels of GIRK1 in sections from GIRK3 knock-out mice, possibly indicating a low-level expression of GIRK3 in the spinal cord dorsal horn (Fig. 1G). In

contrast, the pattern and intensity of GIRK2 staining in GIRK3 knock-out sections was indistinguishable from that observed in wild-type sections (Fig. 1H).

GIRK subunit expression in the superficial layers of the dorsal horn suggested that spinal G-protein-gated K$^+$ channels could modulate nociception and morphine analgesia. To test this hypothesis directly, we compared the performance of wild-type mice with GIRK knock-out mice in the tail-flick test of thermal nociception, using intrathecal injection of morphine to achieve a degree of regional specificity with regard to drug action. Because the relevance of spinal G-protein-gated K$^+$ channels to nociception and morphine analgesia was unknown, both baseline tail-flick responses and postinjection responses were measured at 49°C, 52.5°C, and 55°C. These temperatures stimulate different complements of primary afferent fibers and evoke baseline response latencies of 2–7 sec in wild-type mice. The shorter latency responses evoked by higher temperatures (55°C) are thought to reflect the function of spinal reflexes, whereas responses evoked by lower temperatures (49°C) likely involve significant supraspinal integration (Le Bars et al., 2001).

Analysis of baseline tail-flick latencies at 49°C revealed main effects of both gender ($F_{(1,494)} = 7.2$; $p < 0.05$) and genotype ($F_{(4,494)} = 54.9$; $p < 0.05$), as well as an interaction between the two factors ($F_{(4,494)} = 2.6$; $p < 0.05$). Indeed, wild-type male mice exhibited significantly longer baseline latencies than wild-type females at 49°C (7.21 ± 0.17 vs 6.13 ± 0.15 sec; $p < 0.01$) (Fig. 2A). Tail-flick responses of GIRK3 knock-out mice were indistinguishable from their wild-type counterparts, and comparable gender differences were observed in this group as well. Relative to their same-sex wild-type counterparts, however, baseline tail-flick latencies were significantly lower for GIRK1 knock-out males (5.32 ± 0.15 sec; $p < 0.05$) and females (5.05 ± 0.13 sec; $p < 0.05$), as well as for GIRK2 knock-out males (4.71 ± 0.17 sec; $p < 0.05$) and females (4.78 ± 0.14 sec; $p < 0.05$). In addition, GIRK2 knock-out mice exhibited lower baseline latencies than their same-sex GIRK1 knock-out counterparts at all three temperatures, with significant differences observed between the females at 52.5°C and 55°C (Fig. 2B,C). Consistent with previous observations (Mitrovic et al., 2003), gender differences were not observed within the GIRK1 knock-out or GIRK2 knock-out groups at any temperature. Finally, GIRK2/GIRK3 double knock-out mice performed similarly to GIRK2 knock-out mice at all three temperatures, a finding consistent with a minor role for GIRK3 in the measured behavior. The overall pattern of baseline responses at the two higher bath temperatures was primarily consistent with the observations at 49°C, with the exception that, at 55°C, gender differences in the wild-type ($p = 0.21$) and GIRK3 knock-out ($p = 0.73$) groups were not significant (Fig. 2B,C).
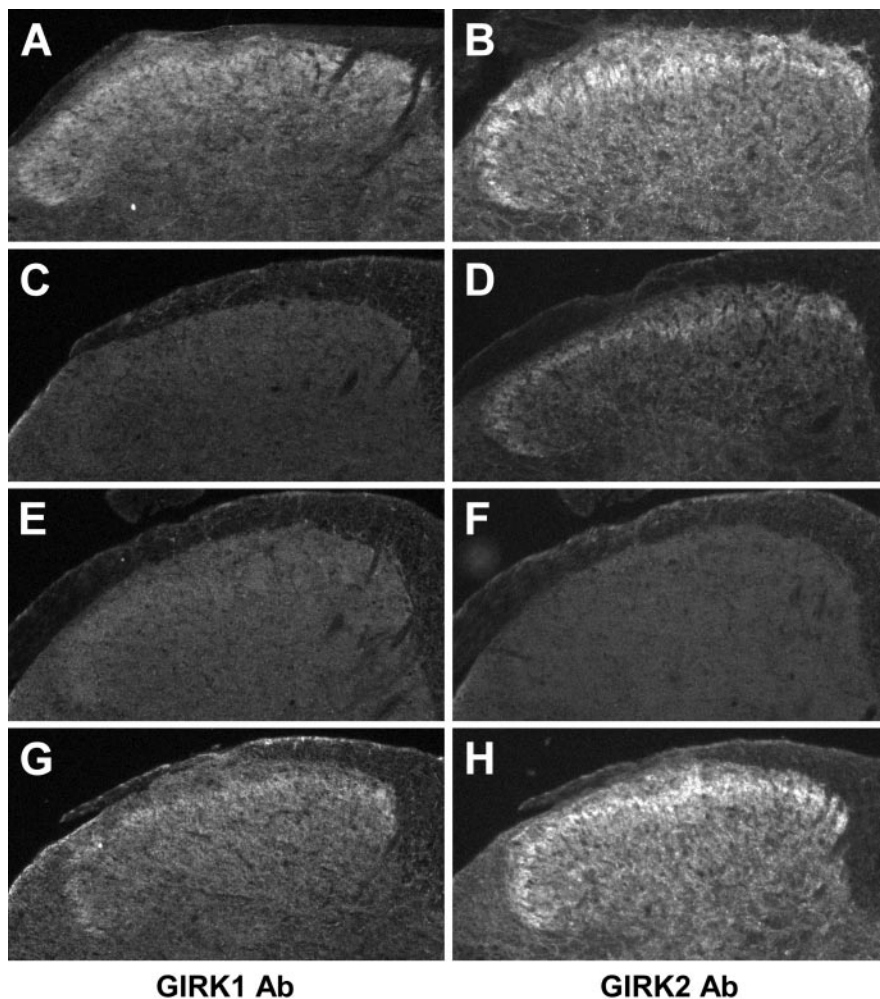

Figure 1. GIRK subunit expression in the dorsal horn of the spinal cord. Spinal cord sections from wild-type (A, B), GIRK1 knock-out (C, D), GIRK2 knock-out (E, F), and GIRK3 knock-out (G, H) mice were stained with anti-GIRK1 (left column) or anti-GIRK2 (right column) antibodies (Ab). Note the absence of GIRK1 and GIRK2 staining in sections from the corresponding knock-out mice (C, F, respectively) and their dramatic reduction in sections from GIRK2 knock-out (E) and GIRK1 knock-out (D) mice, respectively. Also note the slightly lower levels of GIRK1 in sections from GIRK3 knock-out (G), but not GIRK2 knock-out (H), mice. Results are representative of the data obtained from three complete sets of wild-type and GIRK knock-out mice.

After the determination of baseline responses, mice were injected intrathecally with either saline or morphine (0.1, 0.3, 1, and 3 nmol). Postinjection response latencies were measured at 49°C, 52.5°C, and 55°C, and the difference between preinjection and postinjection latencies (ΔTF latency) was calculated for each animal. Analysis of the 49°C data revealed main effects of morphine dose ($F_{(4,494)} = 52.2$; $p < 0.05$) and genotype ($F_{(4,494)} = 2.8$; $p < 0.05$) but no effect of gender ($F_{(1,494)} = 2.0$; $p = 0.16$). Analysis of the 52.5°C and 55°C data revealed similar contributions of dose and genotype, but not gender, to behavioral performance. As such, ΔTF latency data for male and female mice were combined within genotypes at all three temperatures. Morphine increased tail-flick latencies at all three temperatures and in all genotypes tested (Fig. 3). Although GIRK3 knock-out mice behaved indistinguishably from their wild-type counterparts, GIRK1 knock-out and GIRK2 knock-out mice displayed diminished effects of morphine at the two highest morphine doses (1 and 3 nmol) tested (Fig. 3). Indeed, the lower two morphine doses (0.1 and 0.3 nmol) produced equivalent responses in all genotypes, at all three temperatures. The behavior of GIRK2/GIRK3 double knock-out mice was equivalent to that of GIRK1
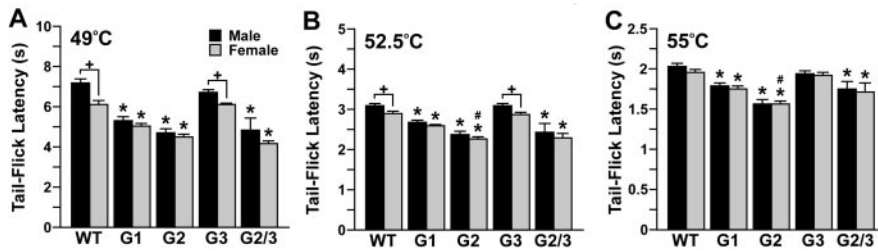
Figure 2. Baseline tail-flick latencies of wild-type and GIRK knock-out mice. Preinjection tail-flick latencies were measured at 49°C (*A*), 52.5°C (*B*), and 55°C (*C*). Both male (black) and female (gray) mice were tested for each genotype. Group designations and sizes (*n* = male, female) are as follows: wild-type (WT; *n* = 83, 85), GIRK1 knock-out (G1; *n* = 52, 46), GIRK2 knock-out (G2; *n* = 23, 45), GIRK3 knock-out (G3; *n* = 64, 83), and GIRK2/GIRK3 double knock-out (G2/3; *n* = 4, 10) mice. Statistical symbols and comparisons are as follows: *$p < 0.05$, GIRK knock-out versus wild type (same gender); +$p < 0.05$, male versus female (same genotype); #$p < 0.05$, GIRK2 knock-out versus GIRK1 knock-out.
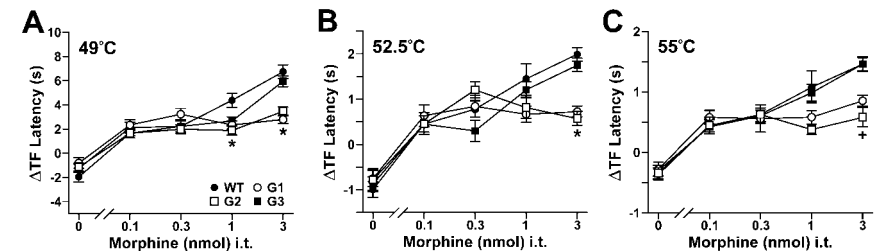

Figure 3. Effect of intrathecal morphine in wild-type and GIRK knock-out mice. Tail-flick latencies were measured at 49°C (*A*), 52.5°C (*B*), and 55°C (*C*), 10 min after the intrathecal injection of saline or morphine (0.1, 0.3, 1, and 3 nmol). The difference between preinjection and postinjection tail-flick latency (ΔTF latency) is plotted as a function of morphine dose. Separate cohorts of mice were used for each drug condition. Wild-type (WT, black circles), GIRK1 knock-out (G1, white circles), GIRK2 knock-out (G2, white squares), and GIRK3 knock-out (G3, black squares) groups were balanced with respect to gender and ranged in size from 11 to 38 animals per dose. Statistical symbols are as follows: *$p < 0.05$, GIRK1 and GIRK2 knock-out versus wild type (WT) (same dose); +$p < 0.05$, GIRK2 knock-out versus wild type (same dose).
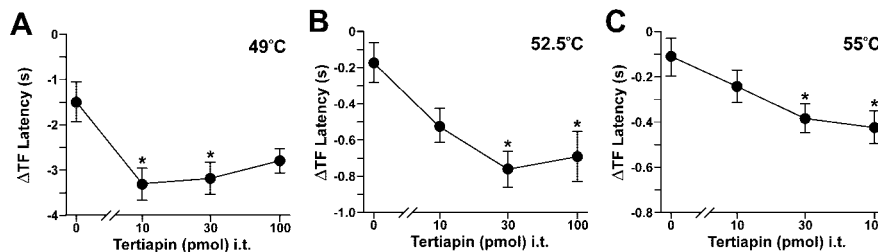

Figure 4. Effect of intrathecal tertiapin in wild-type mice. Tail-flick latencies of wild-type mice were measured at 49°C (*A*), 52.5°C (*B*), and 55°C (*C*) before and after the intrathecal injection of saline or tertiapin (10, 30, and 100 pmol). The difference between preinjection and postinjection tail-flick latency (ΔTF) was calculated and plotted as a function of tertiapin dose. Separate cohorts of mice were used for each tertiapin dose, and groups (26–29 mice each) were balanced with respect to gender. Tertiapin produced dose-dependent decreases in tail-flick latencies at all three temperatures. *$p < 0.05$, tertiapin versus saline.

responses of wild-type C57BL/6 mice. Baseline tail-flick latencies were measured at all three temperatures, followed by the intrathecal injection of either saline or tertiapin (10, 30, and 100 pmol). After a 10 min postinjection interval, tail-flick latencies were measured again, and ΔTF latencies were calculated as described above. A main effect of tertiapin dose ($F_{(3,110)} = 6.0$; $p < 0.01$) but not gender ($F_{(1,110)} = 0.2$; $p = 0.646$) was observed. Intrathecal tertiapin decreased response latencies relative to saline at all doses, with the 30 pmol dose yielding the most consistent and robust effect at the three temperatures tested (Fig. 4).

Tertiapin also blocks members of the Kir1 channel family (Jin and Lu, 1998, 1999; Jin et al., 1999). To determine whether the effect of intrathecal tertiapin was attributable to interaction with non-GIRK targets in the spinal cord, we measured tail-flick latencies in GIRK2 knock-out mice before and after injection of either saline or 30 pmol of tertiapin. No significant differences in ΔTF latency were observed between the GIRK2 knock-out mice injected with saline (*n* = 21) or tertiapin (*n* = 21) at 49°C (−0.35 ± 0.29 vs 0.02 ± 0.17; $p = 0.29$), 52.5°C (−0.19 ± 0.12 vs −0.21 ± 0.11; $p = 0.92$), and 55°C (−0.09 ± 0.08 vs −0.02 ± 0.08; $p = 0.51$), suggesting that the effect of intrathecal tertiapin results primarily from the blockade of spinal G-protein-gated K⁺ channels. Thus, both pharmacological and genetic approaches implicated spinal GIRK channels in the modulation of thermal nociception.

Last, we asked whether tertiapin could blunt the analgesic effect of morphine when both substances were coadministered by intrathecal injection. Wild-type C57BL/6 mice were given a range of morphine doses (0, 0.1, 0.3, 1, and 3 nmol) together with either saline or 30 pmol of tertiapin. Drug-induced changes in tail-flick latencies were normalized to offset the acute hyperalgesic effect of tertiapin, thus permitting a direct compari-

knock-out and GIRK2 knock-out mice (data not shown), arguing that GIRK3-containing channels contribute little, if anything, to the analgesic effect of intrathecal morphine.

GIRK2 knock-out mice display numerous phenotypes ranging in severity from hyperactivity to decreased anxiety to spontaneous seizures (Signorini et al., 1997; Blednov et al., 2001b). Although GIRK1 knock-out mice are relatively less affected than GIRK2 knock-out mice (C. L. Marker, K. D. Wickman, unpublished observations), one concern is that unpredictable compensation, developmental abnormalities, and/or alterations in functionally relevant supraspinal and spinal neuronal circuitry may have occurred, which could confound the accurate assessment of spinal G-protein-gated K⁺ channel contributions to nociception. To address this issue, we measured the effect of the G-protein-gated K⁺ channel blocker tertiapin on the tail-flick

son of morphine effects between the saline- and tertiapin-treated groups (see Materials and Methods). Reminiscent of the observations involving the GIRK1, GIRK2, and GIRK2/GIRK3 knock-out mice, the lower morphine doses produced equivalent antinociception in both the saline and tertiapin groups, whereas the analgesia evoked by the highest morphine dose (3 nmol) was markedly reduced in tertiapin-treated mice at all three temperatures (Fig. 5). As such, tertiapin-treated wild-type mice mirror the behavior of GIRK1 knock-out and GIRK2 knock-out mice with respect to morphine analgesia.

Discussion

The goal of this study was to clarify the contribution of spinal G-protein-gated K⁺ channels to thermal nociception and morphine analgesia. We demonstrated that GIRK1 and GIRK2 sub-

unit expression is enriched in the superficial layers of the spinal cord dorsal horn and that the genetic ablation of either subunit was correlated with dramatic reductions in the expression of the other. Consistent with the localization data, GIRK1 knock-out and GIRK2 knock-out mice exhibited thermal hyperalgesia in the tail-flick test and displayed diminished analgesic responses to the two higher doses of intrathecal morphine tested compared with wild-type controls. The lack of phenotype observed in GIRK3 knock-out mice and the similarity in the phenotypes of GIRK2 knock-out and GIRK2/GIRK3 double knock-out mice argue that GIRK3-containing channels play little role in the behavioral responses measured in this study. Finally, we demonstrated that intrathecal injection of the G-protein-gated K⁺ channel blocker tertiapin produced hyperalgesia and decreased the effect of coadministered morphine in wild-type mice, in a manner consistent with our observations of GIRK1 knock-out and GIRK2 knock-out mice. We conclude that spinal G-protein-gated K⁺ channels, consisting primarily of GIRK1/GIRK2 complexes, modulate thermal nociception and mediate a significant component of the analgesia observed with high morphine doses.

Although previous efforts have implicated GIRK2-containing channels in nociception and antinociception, this is the first study to directly probe the involvement of GIRK1 in these processes. The overlap in expression of GIRK1 and GIRK2 in the superficial layers of the spinal cord dorsal horn suggested that a physical interaction exists between the subunits, a hypothesis supported by the observations that GIRK1 immunoreactivity was virtually absent in the dorsal horn of GIRK2 knock-out spinal cord sections and that GIRK2 immunoreactivity was dramatically reduced in sections from GIRK1 knock-out mice. Given that phenotypes observed in both the GIRK1 knock-out and GIRK2 knock-out mice were nearly identical, it seems reasonable to conclude that GIRK1/GIRK2 channels represent the dominant type of G-protein-gated K⁺ channel in the superficial layers of the spinal cord dorsal horn. Interestingly, the patterns of GIRK1 and GIRK2 immunoreactivity did not overlap perfectly in other regions of the spinal cord, suggesting that multiple pools of G-protein-gated K⁺ channels of distinct subunit composition exist and may contribute in unique manners to information processing and relay within the spinal cord.

Frequent criticisms of constitutive gene ablation studies relate to the myriad potential compensatory processes that could be triggered by the absence of the targeted gene throughout development, as well as subtle interactions that could occur between the targeted gene, genetic background, and environment (Mogil et al., 1996; Mogil, 1999). We attempted to address these concerns using multiple approaches. The knock-out lines used in this study were congenic in nature, having been backcrossed for 9–20 rounds against the well characterized, C57BL/6 mouse strain. Phenotypes observed in the GIRK1 knock-out and GIRK2 knock-out strains are not easily attributed to a small residual component of 129/Sv genetic content, because the similarly backcrossed GIRK3 knock-out line behaved indistinguishably from wild-type counterparts in this study. Furthermore, the behavioral data are consistent with the GIRK subunit expression profile in the spinal cord. Finally, the overall similarity in the performance of tertiapin-treated wild-type mice and the GIRK1 knock-out
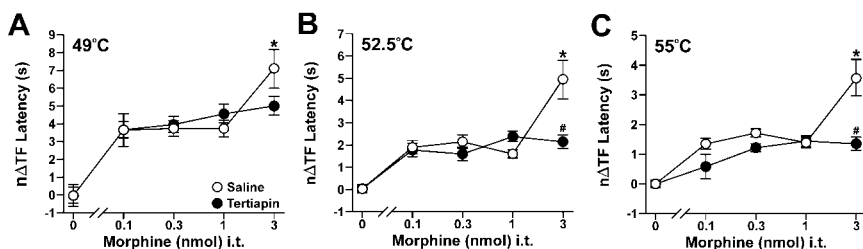

Figure 5. Tertiapin blunts the analgesic effect of high-dose morphine in wild-type mice. The analgesic effect of morphine (0, 0.1, 0.3, 1, and 3 nmol) coadministered intrathecally with either saline (white circles) or 30 pmol of tertiapin (black circles) was measured in wild-type mice. Preinjection and postinjection tail-flick latencies were measured at 49°C (A), 52.5°C (B), and 55°C (C), and normalized ΔTF latency (nΔTF latency) was calculated as described (see Materials and Methods). Separate cohorts of mice were used for each drug condition. Groups were balanced with respect to gender, and sizes ranged from 11 to 16 mice per drug condition. *$p < 0.05$, tertiapin versus saline; **$p < 0.05$, 3 versus 1 nmol of morphine.

and GIRK2 knock-out mice suggests that the phenotypes observed in the GIRK knock-out mice do not reflect dramatic developmental alterations or compensations resulting from constitutive gene ablation.

Interestingly, we observed a decreased analgesic effect of intrathecal morphine in GIRK knock-out and tertiapin-treated wild-type mice, but only at the higher morphine doses tested. There are several potential explanations for these observations. Although a recent study involving MOR knock-out mice implicated this receptor subtype in the analgesic action of intrathecal morphine (Guo et al., 2003), the decreased maximal efficacy of morphine observed in our study may relate to the actions of morphine at other receptors. Morphine at high concentrations can activate both δ-opioid (DORs) and κ-opioid (KORs) receptors (Law et al., 2000; Williams et al., 2001), and the activation of both receptors in the spinal cord evokes analgesia in mice (Improta and Broccardo, 1992; Chien et al., 1994; Eckert and Light, 2002). Furthermore, DOR and KOR modulation of G-protein-gated K⁺ channels has been reported previously in heterologous expression systems (Henry et al., 1995; Kobayashi et al., 1996; Kovoor et al., 1997). Accordingly, the disruption of KOR- and/or DOR-dependent signaling would be predicted to impact the maximal analgesic efficacy of morphine, whereas effects at low doses would be preserved. It is also possible that our observations reflect the differential potencies of morphine with respect to the modulation of individual downstream effectors such as adenylyl cyclase, voltage-gated Ca²⁺ channels, voltage-gated K⁺ channels, and G-protein-gated K⁺ channels (Law et al., 2000). Hence, the decreased analgesic effect of morphine observed at higher doses in GIRK knock-out and tertiapin-treated wild-type mice could indicate that G-protein-gated K⁺ channels are less sensitive to morphine than other effectors.

Another possible explanation for the decreased efficacy of morphine observed in our mouse models is that morphine is a more potent trigger of presynaptic antinociceptive mechanisms, whereas postsynaptic mechanisms are required for its maximal analgesic effect. Unmyelinated C fibers and myelinated Aδ primary afferent fibers are thought to underlie hot-plate and tail-flick responses evoked by temperatures ranging from 49°C to 55°C (Millan, 1999, 2002). MORs are expressed on presynaptic terminals of C fibers and on secondary dorsal horn neurons responsive to both C and Aδ fiber input (Aicher et al., 2000). This and other lines of evidence argue that MOR-preferring opiates such as morphine evoke analgesia via the presynaptic inhibition of C fibers and by blunting the excitatory postsynaptic responses of secondary dorsal horn neurons to C and Aδ fiber afferent input

(Yaksh, 1997, 1999). Because available evidence indicates that G-protein-gated K$^+$ channels are postsynaptic effectors in the nervous system (Luscher et al., 1997; Slesinger et al., 1997), our findings suggest that postsynaptic inhibition of secondary dorsal horn neurons contributes primarily to the analgesia caused by high doses of intrathecal morphine.

G-protein-gated K$^+$ channels are poised to impact nociception at multiple anatomic levels. A recent report identified GIRK2-containing channels on nociceptive primary afferent neurons in peripheral tissue and implicated G-protein-gated K$^+$ channels in an endogenous peripheral analgesic cascade (Khodorova et al., 2003). In addition, GIRK subunit mRNAs have been detected in critical supraspinal centers involved in descending modulation and information integration, including the thalamus and PAG (Karschin et al., 1996). Indeed, a role for supraspinal G-protein-gated K$^+$ channels in nociception and morphine analgesia may be inferred from comparisons between this and our previous study. GIRK3 knock-out mice exhibited thermal hyperalgesia in the hot-plate test, whose endpoint (pawlick) requires supraspinal integration of the input stimulus (Le Bars et al., 2001; Marker et al., 2002). Because GIRK3 knock-out mice behaved indistinguishably from wild-type mice in the tailflick assay, which for the short latency responses does not require supraspinal integration (Le Bars et al., 2001), it seems reasonable to suggest that GIRK3-containing channels may contribute to the tone of descending modulatory pathways or supraspinal pain integration centers.

In conclusion, we present evidence that spinal G-protein-gated K$^+$ channels modulate thermal nociception and contribute to the analgesic effect of morphine. It will be important to clarify whether and how G-protein-gated K$^+$ channels contribute to pain processing associated with noxious mechanical and chemical stimuli. These findings could have considerable clinical implications, because the spinal delivery of a direct G-protein-gated K$^+$ channel agonist could represent a highly selective pharmacotherapy for certain types of pain.

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