Metabotropic glutamate receptor 5 regulates excitability and Kv4.2-containing K^+ channels primarily in excitatory neurons of the spinal dorsal horn

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Submitted 3 December 2010; accepted in final form 30 March 2011

METHODS

Animals. All 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 (St. Louis, MO). Homozygotic GAD67-GFP [GFP-expressing inhibitory neurons (GIN)] friend virus B-type (FVB) mice, which express enhanced GFP under control of the promoter for the GABA-synthesizing enzyme GAD67 (Oliva et al. 2000), and wild-type FVB mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

Cell culture. Primary cultures of spinal cord superficial dorsal horn neurons were prepared from 3- to 5-day-old GIN or wild-type FVB mouse pups, as previously described (Hu and Gereau 2003; Hu et al.

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Twenty cultures were made for electrophysiology recordings, and 10 cultures were used for double-staining.

**Electrophysiological recording.** Standard whole-cell recordings were made at room temperature using an EPC10 amplifier and Pulse version 8.62 software (HEKA Electronik, Lambrecht, Germany) as previously described (Hu and Gereau 2003; Hu et al., 2003). Electrode resistances were 3–6 MΩ with series resistances of 6–15 MΩ, which were compensated >60%. Only neurons with a resting membrane potential more hyperpolarized than −50 mV were used. All neurons had leak currents <100 pA (at −80 mV), which were not subtracted online. Access resistance and input resistance were monitored by hyperpolarizing current injection throughout the course of the experiment. The data were rejected if either of these parameters changed >20%.

For voltage-clamp recordings in cultured neurons, the bath solution was HBSS containing 500 nM TTX and 2 mM CoCl2 to block voltage-gated Na+ currents and Ca2+ currents. The electrode solution contained (in mM) 140 KCl, 1 MgCl2, 0.5 CaCl2, 5 EGTA, 10 HEPES, 3 Na2ATP, 0.3 Na2GTP, pH 7.4. The membrane voltage was held at −80 mV, and transient potassium currents (ITx) were isolated by a two-step voltage protocol as previously described (Hu and Gereau 2003; Hu et al., 2003). Briefly, a total outward current was evoked by a command potential of +40 mV from a holding potential of −80 mV. The A-type current was dissected away from the sustained current by the voltage protocol (a 150 ms prepulse to −10 mV allowed the transient channels to inactivate, leaving only the sustained current). Subtraction of the sustained current from the total current isolated the A-type current. With the use of this protocol, if the subtraction current is ≥200 pA or the A-type current density is ≥50 pA/pF, it is considered as an A-type current. To determine the voltage dependence of activation, voltage steps of 500 ms were applied at 5-s intervals in +10–mV increments from −80 mV to a maximum of +70 mV. To determine the voltage dependence of inactivation, conditioning pulses ranging from −100 mV to +40 mV were applied in +10 mV increments for 150 ms followed by a step to +40 mV for 500 ms. For current-clamp recording, the intracellular solution contained (in mM) 140 KMeSO4, 2 MgCl2, 1 EGTA, 10 HEPES, 3 Na2ATP, 0.3 Na2GTP, pH 7.4. The bath solution for cultures was HBSS. Action potentials (APs) were generated by current injection from a holding potential of −70 mV. Throughout the recording, the holding potential was maintained by current injection. Intracellular excitability was measured every 15 s using a constant amplitude small depolarizing pulse (800 ms, 10–80 pA). The amplitude that evoked 2–8 APs during the baseline period was selected and remained constant throughout the recording. The first spike latency was measured as the time between the stimulus onset and the first spike. The spike frequency was measured by counting the number of spikes within a depolarizing pulse of 800 ms.

**Immunofluorescence.** FVB wild-type mice were anesthetized ip with sodium pentobarbital (60 mg/kg) and perfused transcardially with 4% paraformaldehyde phosphate buffer (PB) solution. Lumbar spinal cords were dissected out and postfixied overnight in 4% paraformaldehyde at 4°C, followed by cryoprotection in 30% sucrose/PB at 4°C. Coronal sections (30 μm) were cut with a cryostat (Leica, Nussloch, Germany) and were washed several times in 0.1 M PBS. All incubations and washes were performed at room temperature unless otherwise noted. Sections were then blocked in 3% normal goat serum (Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100 in 0.1 M PBS (3% NGST) for 1 h. Primary antibodies were diluted in 3% NGST. Sections were incubated in a mixture of mouse anti-Kv4.2 (1:100; Antibodies Incorporated, Davis, CA) and rabbit polyclonal antivesicular glutamate transporter 2 (VG2T; 1:1,000; a generous gift from Dr. Robert H. Edwards, University of California, San Francisco, CA) or rabbit polyclonal anti-PKCγ (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Sections were rinsed with 3% NGST three times for 10 min each and were blocked in 10% NGST for 1 h, followed by incubation in a mixture of secondary goat anti-rabbit cyanine 5 (Cy5)-conjugated antibody (1:1,100; Zymed Laboratories, San Francisco, CA) and goat antimouse Cy3-conjugated antibody (1:1,000; Zymed Laboratories) in 10% NGST. The sections were rinsed in PBS and observed under a confocal laser-scanning microscope (Olympus Optical, Tokyo, Japan).

Cultured spinal cord neurons were fixed with 2% paraformaldehyde for 5 min and further fixed with 4% paraformaldehyde for 10 min at 4°C. Cells were rinsed with 0.1 M PBS four times for 5 min each and then blocked in NGST for 1 h. All primary antibodies were diluted in 3% NGST. Cells were incubated in rabbit polyclonal anti-mGlur5 (Upstate Biotechnology, Lake Placid, NY) 1:1,600 or mouse anti-Kv4.2 (Antibodies Incorporated) 1:100 at 4°C overnight. Cells were rinsed with 3% NGST three times for 5 min each and were blocked in 10% NGST for 1 h, followed by incubation in secondary goat anti-rabbit Cy3-conjugated antibody (Zymed Laboratories) 1:1,000 or goat anti-mouse Cy3-conjugated antibody (Zymed Laboratories) 1:1,000 in 10% NGST. Cells were then rinsed in PBS and observed under a confocal laser-scanning microscope (Olympus Optical). For double-staining of mGlur5 and neuronal nuclei (NeuN), Kv4.2 and NeuN, or VG1T, or VG2T, or neurokinin 1 (NK1), or PKCγ, the same procedure described above was used except for the antibodies. Briefly, cells were incubated in a mixture of mouse anti-Kv4.2 (Antibodies Incorporated) 1:200 and rabbit polyclonal VG1T (a generous gift from Dr. Robert H. Edwards) 1:1,000 or VG2T 1:1,000 or NK1 (Chemicon, El Segundo, CA) 1:1,000 or PKCγ 1:1,000 primary. After washing, the cells were incubated in a mixture of secondary anti-rabbit Alexa-488-conjugated IgG (Molecular Probes, Eugene, OR) 1:2,000 and anti-mouse Cy3-conjugated antibody 1:1,000 at room temperature.

**Drug application.** (RS)-3,5-Dihydroxyphenylglycine (DHPG) was purchased from Tocris Bioscience (Ballwin, MO) and dissolved in HBSS for bath application. DHPG (100 μM) was applied for 3 min.

**Data analysis.** Offline evaluation was done using Pulse v8.62 software or PatchMaster (HEKA Elektronik) and Origin 7.1 or 8.1 (OriginLab, Northampton, MA). Data are expressed as original traces or as mean ± SE. The voltage dependence of activation and inactivation of IA was fitted with the Boltzmann function. For activation, peak currents were converted to conductance (G) by the formula G = I/Vm(Vm − V1/2) and 1/s is the current. V1/2 is the membrane voltage of depolarization pulses, and V1/2 is the calculated potassium reversal potential (−84 mV). The function G/Gmax = 1/I(1 + exp(V1/2 − V/k)) was used to normalize conductance, where Gmax is the maximal conductance obtained with a depolarizing pulse to +70 mV, V1/2 is the half-maximal voltage (V), and k is the slope factor. For inactivation, I(t)/I(t) = 1/(1 + exp(V1/2 − V/k)) was used, where I(t) is the maximal current obtained with a −100 mV prepulse. The decay phase of A-type currents was fitted with a double-exponential equation [y = A1/exp(−x/t1) + A2/exp(−x/t2) + y0], where t1 and t2 were the decay time constants, and A1 and A2 were the proportions of each phase, respectively.

For all experiments, treatment effects were statistically analyzed by paired or two-sample Student’s t-test. The distribution of firing patterns was statistically analyzed with a χ2 test. Error probabilities of P < 0.05 were considered statistically significant.

**RESULTS**

**Expression of Kv4.2 in GAD67-GFP neurons.** In previous studies, we found that a small portion of dorsal horn neurons does not have Kv4.2-mediated A-type currents (data not shown). To determine the percentage of Kv4.2-expressing neurons in dorsal horn cultures, we used NeuN as a neuronal marker and performed double-immunofluorescence staining for Kv4.2 and NeuN. Kv4.2 was exclusively expressed in neurons. Three hundred thirty-six out of 396 (85%) neurons
were Kv4.2 positive (Fig. 1). Next we asked what subtype of neurons expresses Kv4.2. We took advantage of a transgenic mouse expressing GFP driven by the GAD67 promoter [GAD67-GFP or GIN mice (Oliva et al. 2000)] to examine Kv4.2 expression in GAD67-GFP neurons, which represent >67% of GABAergic inhibitory neurons (Dougherty et al. 2005, 2009). In the present study, 64 GAD67-GFP-expressing neurons were examined (note that only cells in the field where we saw GFP neurons were counted); 44 of these GAD67-GFP neurons also expressed Kv4.2. Thus 69% of GAD67-GFP neurons were Kv4.2 positive (Fig. 1). We also randomly counted 125 Kv4.2-positive cells, and only four of these neurons were GAD67-GFP positive. These results suggest that a very small proportion (3.2%) of Kv4.2-expressing neurons is GAD67-GFP positive, but among the GAD67-GFP neurons, most are Kv4.2 positive.

Characterization of A-type currents in spinal dorsal horn GFP-expressing neurons. Following the observation described above, we asked whether Kv4.2 is functionally expressed in GAD67-GFP neurons. To answer this question, we performed voltage-clamp recordings in cultured spinal dorsal horn neurons expressing GFP and non-GFP neurons from GIN (GAD67-GFP) mouse pups. We found that the membrane capacitance and resting membrane potential were similar in both groups (data not shown). A-type currents from GIN transgenic mice were similar to those from wild-type FVB mice. Interestingly, A-type currents were much smaller (43.2%), whereas sustained currents were significantly larger (33.5%) in GFP-expressing neurons relative to the non-GFP neurons (Fig. 2). There was no difference in total outward I_A between these two groups (Fig. 2B). Some GFP neurons (six out of 71) had no or very small A-type currents (≤200 pA). Most GFP neurons (53 out of 71; 75%) exhibited slowly decaying A-type currents best fit with a single time constant (190 ± 27 ms; n = 16) at a depolarizing step of +40 mV (Fig. 2A) and slower recovery from steady-state inactivation with only <75% recovery from inactivation after 800 ms (Fig. 3B). Non-GFP neurons (37 out of 42; 88%) from the same cultures expressed A-type currents with fast activation and rapid inactivation best fit with two time constants (τ_fast of 20 ± 1 ms and τ_slow of 298 ± 24 ms; n = 13; Fig. 2A) and >90% recovery from inactivation after 800 ms (Fig. 3B). Activation curves of A-type currents from GAD67-GFP neurons were significantly right-shifted from −7.0 ± 1.5 mV (n = 10) to +1.8 ± 2.1 mV (n = 13; P < 0.05), but the slope of the activation curve was not markedly different (Fig. 3A). In addition, steady-state inactivation curves in GAD67-GFP neurons were significantly shifted from −56.0 ± 1.0 mV (n = 15) to −35.6 ± 4.1 mV (n = 25; P < 0.05); the slope of the inactivation curve in these neurons was also altered from 8.5 ± 0.4 (n = 15) to 16.0 ± 2.3 (n = 25; P < 0.05; Fig. 3A). All of these differences in the properties of A-type currents in GAD67-GFP neurons relative to non-GFP neurons indicate that the majority of these GABAergic neurons expresses little or no Kv4.2-mediated A-type currents, whereas Kv4.2-like A-type currents are much more robustly expressed in the non-GFP population of dorsal horn neurons.

Intrinsic membrane properties in GAD67-GFP neurons. We and others (Heinke et al. 2004; Hu and Gereau 2003; Ruscheweyh and Sandkuhler 2002) have previously identified at least four categories of dorsal horn neurons based on firing patterns. In the present study, a total of 108 cultured dorsal horn neurons from GIN mice was examined. AP firing patterns from GIN transgenic mice were similar to those from wild-type FVB mice. The mean rheobase in

Fig. 1. Colocalization of Kv4.2 with neuronal nuclei (NeuN) and expression of Kv4.2 in green fluorescent protein under the control of the glutamic acid decarboxylase 67 promoter (GAD67-GFP)-expressing dorsal horn neurons. A: representative confocal images of Kv4.2 (red) and NeuN (green) immunofluorescent staining in cultured dorsal horn neurons. The overlap of Kv4.2 and NeuN immunofluorescence is shown in yellow in the right panel titled “Merge”. B: representative confocal images of Kv4.2 (red) in GAD67-GFP-expressing dorsal horn neurons (green). The overlap of Kv4.2 immunofluorescence and GFP fluorescence is indicated with arrows in the right panel. Original scale bars: 40 μm.
GAD67-GFP neurons (47.0 ± 3.5 pA; n = 47) was not significantly different from that in non-GFP neurons (42.4 ± 6.2 pA; n = 27; P > 0.05). The neurons could be divided into five categories, according to their firing patterns when depolarized from a holding potential of −70 mV (Fig. 4): 1) phasic firing (the spike amplitude was markedly attenuated at the end of strong depolarizing steps); 2) repetitive firing (no spike amplitude attenuation at higher current injection); 3) irregular firing pattern (including gap firing; we previously combined this group with the repetitive firing group (Hu and Gereau 2003)); 4) delayed firing (a delay between the onset of current injection and the first AP); and 5) single spike (even at strong depolarization). Similar to our previous study (Hu and Gereau 2003), non-GFP neurons showed an approximately equal distribution of phasic, repetitive, delayed, and irregular firing pattern (21–23%). When only considering GAD67-GFP neurons, the distribution of firing patterns was significantly different (P < 0.05; χ² test) with a decrease in the proportion of phasic and delayed firing patterns and an increase in the proportion of repetitive and irregular firing patterns (Fig. 4).

Activation of mGlu5 does not modulate A-type currents or neuronal excitability in GAD67-GFP-expressing neurons. We have shown previously that activation of mGlu5 by DHPG inhibits Kv4.2-mediated A-type currents and increases neuronal excitability in spinal dorsal horn neurons (Hu et al. 2007). As demonstrated above, the GAD67-GFP-expressing neurons express very little Kv4.2-mediated A-type current. We sought to test whether DHPG modulates A-type currents and neuronal excitability in this subgroup, GABAergic neurons. We performed both voltage-clamp and current-clamp recordings and evaluated the DHPG effect on A-type currents and neuronal excitability in GAD67-GFP-expressing and non-GFP neurons. In voltage-clamp recordings, bath application of 100 μM DHPG for 3 min had no effect on A-type currents in GAD67-GFP neurons, whereas DHPG decreased A-type currents in non-GFP neurons, as we reported previously (Fig. 5, A and B). In current-clamp recordings, we recorded APs from neurons with different firing patterns (except single spike). DHPG decreased first spike latency and increased spike frequency in non-GFP neurons but had no significant effect on firing properties of GAD67-GFP neurons (Fig. 5, C and D). These results
demonstrate that mGlu5-Kv4.2 signaling is not associated with this type of GABAergic neurons.

Expression of mGlu5 in GAD67-GFP neurons. As we showed above, DHPG did not modulate A-type currents or neuronal excitability in GAD67-GFP neurons. We next asked whether mGlu5 protein is expressed in GAD67-GFP neurons. We performed immunofluorescence staining for mGlu5 in dorsal horn neurons cultured from GIN pups. Consistent with previous studies in other labs (Edling et al. 2007; Janssens and Lesage 2001), mGlu5 was not only expressed in neurons but also expressed in glial cells in the dorsal horn. A total of 280 NeuN-positive cells (neurons) and 242 mGlu5-positive cells was examined. One hundred ninety-two cells coexpressed NeuN and mGlu5. Thus 68.6% of neurons expressed mGlu5, and 78.3% mGlu5-positive cells were neurons (Fig. 6). We next examined the coexpression of mGlu5 and GAD67-GFP: a total of 222 mGlu5-positive cells was examined; 36 of these neurons expressed both mGlu5 and GAD67-GFP. Thus 16% of mGlu5 cells also expressed GAD67-GFP (Fig. 6). We also randomly counted 140 mGlu5-positive cells and found that only 10 of 140 (7%) of these cells expressed GAD67-GFP. These results suggest that mGlu5 protein is only minimally expressed in the GAD67-GFP-expressing population of inhibitory neurons.

Coexpression of Kv4.2 and vesicular glutamate transporters. Since only 3.2% of Kv4.2-positive neurons express GAD67-GFP, we hypothesized that the majority of Kv4.2-positive neurons includes excitatory neurons. It has been shown that VGT1 or VGT2 is selectively expressed in glutamatergic neurons (Bellocchio et al. 1998; Brumovsky et al. 2007; Fujiyama et al. 2001; Ponzio et al. 2006; Todd et al. 2003). We performed double-immunofluorescence staining for Kv4.2 and VGT1 or VGT2. Both VGT1 and VGT2 were detected in most cultured dorsal horn neurons. Sixty-eight out of 73 (93%) Kv4.2-positive neurons were VGT1 positive; 67 out of 70 (96%) VGT1 neurons were Kv4.2 positive (Fig. 7A). Seventy-

Fig. 3. The kinetic profile of A-type currents in GAD67-GFP-expressing dorsal horn neurons and non-GFP neurons from GIN mice. A: steady-state activation and inactivation curves from non-GFP neurons (n = 13) or GAD67-GFP-expressing neurons (n = 15). B: recovery from inactivation of A-type currents in non-GFP (n = 15) or GAD67-GFP-expressing (n = 25) neurons. C: representative recordings from non-GFP and GAD67-GFP-expressing neurons showing recovery from inactivation of A-type currents in these cells. Inset: protocol for recovery from inactivation, where first duration of voltage step from 40 to −80 mV is 3 ms; incremental duration, 20 ms.
seven out of 88 (88%) Kv4.2-positive neurons were VGT2 positive; 76 out of 80 (95%) VGT2 neurons were Kv4.2 positive (Fig. 7A). Thus both VGT1 and VGT2 proteins are highly colocalized with Kv4.2. To further confirm Kv4.2 colocalization with VGT2, we double-labeled spinal cord sections from adult mice with antibodies against Kv4.2 and VGT2. Confocal images showed that there was a significant overlap between the distributions of Kv4.2 and VGT2 in the superficial dorsal horn (Fig. 7B). These results suggest that the majority of Kv4.2-expressing neurons includes glutamatergic neurons in the spinal dorsal horn.

Coexpression of Kv4.2 and NK1 or PKCγ. It has been demonstrated that NK1 and PKCγ are markers of excitatory neurons in the spinal cord dorsal horn (Littlewood et al. 1995; Polgar et al. 1999) and modulators of nociception. We sought to examine Kv4.2 colocalization with these two proteins to further determine the neurochemical phenotype of Kv4.2-expressing neurons. We performed double-immunofluorescence staining for Kv4.2 and NK1 or PKCγ. Both NK1 and PKCγ were widely expressed in cultured dorsal horn neurons.

One hundred eleven out of 140 (86%) Kv4.2-positive neurons were NK1 positive; 111 of 132 (84%) NK1 cells were Kv4.2 positive (Fig. 8A). Seventy of 82 (85%) Kv4.2-positive neurons were PKCγ positive, and 100% of 70 PKCγ-positive neurons were Kv4.2 positive (Fig. 8B). Both NK1 and PKCγ proteins were highly colocalized with Kv4.2. Consistent with this finding, immunofluorescence staining of adult mouse spinal cord slices revealed that Kv4.2 was overlapped with NK1 and PKCγ in the dorsal horn (Fig. 8B).

**DISCUSSION**

In the present study, we demonstrate that the majority of Kv4.2-expressing neurons includes excitatory neurons. This result is consistent with the recent finding showing that A-type currents are associated with excitatory dorsal horn lamina II neurons (Yasaka et al. 2010). Our immunocytochemistry data showed that a very limited number of Kv4.2-expressing neurons are GAD67-GFP positive, whereas the majority of Kv4.2-expressing neurons expresses VGT1/2, which are markers of...
excitatory neurons. This suggests that the majority of Kv4.2-expressing neurons is glutamatergic. However, a significant proportion of GAD67-GFP neurons is Kv4.2 positive, indicating that Kv4.2 is also expressed in some inhibitory neurons. Interestingly, electrophysiological data reveal that Kv4.2-mediated A-type currents are not functionally expressed in the majority of GAD67-GFP neurons, which show either no A-type currents or A-type currents with slower kinetics, suggesting minimal contribution of Kv4.2. This discrepancy may be the result of an insufficient trafficking of Kv4.2 protein to the membrane of these neurons. Another possibility is that Kv4.2 is expressed in the cell surface membrane with very low density and thus contributes only very minimally to whole-cell K⁺ currents. Immunostaining provides information regarding protein expression, which does not necessarily correlate with channel functional expression (Li et al. 2011; Schultz et al. 2007).

We have reported that genetic deletion of Kv4.2 decreases rheobase and increases excitability of dorsal horn neurons (Hu et al. 2006). In this study, we did not observe any differences

Fig. 5. (R,S)-3,5-Dihydroxyphenylglycine (DHPG)-induced modulation of A-type currents and neuronal excitability is absent in GAD67-GFP-expressing neurons. A: representative traces of A-type currents recorded before (Pre-DHPG) and 3 min after application of 100 μM DHPG to non-GFP or GAD67-GFP-expressing neurons in the same preparations. B: summary of the effects of DHPG on peak amplitude of A-type currents in non-GFP (n = 7) or GAD67-GFP-expressing neurons (n = 9). Values represent mean ± SE; ***P < 0.001; 2-sample Student’s t-test. C: representative APs recorded in non-GFP or GAD67-GFP-expressing neurons in cultures from GIN mice before and 3 min after application of 100 μM DHPG. D: summary of changes in first spike latency and spike frequency induced by DHPG in non-GFP (n = 7) or GAD67-GFP neurons (n = 9) from GIN mice. Values represent mean ± SE; *P < 0.05; ***P < 0.001; paired Student’s t-test.
in rheobase in GAD67-GFP neurons relative to non-GFP neurons, although A-type currents are relatively smaller in the GAD67-GFP neurons. Since the delayed rectifier currents (sustained currents) are significantly larger in the GAD67-GFP neurons relative to non-GFP neurons, total $I_A$ was similar in GAD67-GFP neurons compared with non-GFP neurons, suggesting that total $I_A$ (not just A-type currents) likely contributes to setting the rheobase. We also characterized the firing patterns of GAD67-GFP neurons and find that these neurons have decreased prevalence of phasic and delayed firing patterns and an increase in the prevalence of repetitive and irregular firing patterns relative to non-GFP neurons in the same culture preparations. These differences that we observed in the firing properties of GAD67-GFP neurons relative to non-GFP neurons are similar to what we observed in dorsal horn neurons from Kv4.2 knockout mice relative to wild-type controls (Hu et al. 2006). While we propose that the essential lack of Kv4.2-mediated A-type currents in GAD67-GFP neurons, relative to non-GFP neurons, contributes to these differences in AP firing patterns, there are, of course, many other possible mechanisms by which firing properties can be altered.

Our electrophysiological results demonstrate that the steady-state activation and inactivation curves are rightward shifted in the GFP population, suggesting that the genes encoding A-type currents in GFP neurons may be different from those in non-GFP neurons. A-type currents can be mediated by several genes, such as Kv1.4, Kv3.4, Kv4.1, Kv4.2, and Kv4.3. GFP neurons lack Kv4.2-like currents but have slowly decaying A-type currents. A previous study from Tsaur’s lab (Huang et al. 2005) revealed that Kv4.3 is expressed in a subset of excitatory interneurons. Ours and Huang’s (Huang et al. 2005) studies suggested that GFP neurons may not express Kv4.2 and Kv4.3. The molecular identity of A-type channels in these dorsal horn neurons remains to be determined.

It is well established that mGlu5 plays important roles in nociceptive plasticity (Fisher and Coderre 1996; Karim et al. 2001). Numerous behavioral studies have demonstrated that mGlu5 in the dorsal horn contributes to inflammatory, visceral, and neuropathic pain (Chen et al. 2000; Fisher et al. 2002; Karim et al. 2001; Montana et al. 2009; Young et al. 1997). We have demonstrated that activation of mGlu5 induces ERK activation, which subsequently inhibits Kv4.2-mediated A-type currents and increases excitability in spinal cord dorsal horn neurons. In the present study, we demonstrate that mGlu5 does not modulate A-type currents or neuronal excitability in GAD67-GFP neurons, whereas in the “non-GFP” population of dorsal horn neurons, this modulation is robust, similar to our previous reports (Hu et al. 2007). This suggests that the mGlu5-Kv4.2 signaling pathway is not operational in this GAD67-GFP population of inhibitory neurons in the dorsal horn. Although immunostaining reveals that one-third of GAD67-GFP neurons expresses mGlu5, it is not surprising that we did not observe the mGlu5-mediated modulation of A-type currents in GAD67-GFP neurons, as the majority of these neurons do not functionally express Kv4.2-mediated A-type currents. The A-type currents observed in 17% of GAD67-GFP neurons may be mediated by other potassium channels, which are not modulated by a mGlu5-activated signal pathway.

We further refined the subpopulations of Kv4.2-expressing neurons by immunocytochemical staining undertaken to examine Kv4.2 coexpression with NK1 and PKC$.\gamma$. It has been proposed that NK1-positive neurons are glutamatergic projection neurons or interneurons in the superficial dorsal horn (Blomeley et al. 2009; Cordero-Erausquin et al. 2004; Jakab et al. 1996; Littlewood et al. 1995). Our results show that Kv4.2 is highly colocalized with NK1, indicating that Kv4.2 modulation in these NK1-expressing neurons could contribute to central sensitization. Indeed, previous reports have suggested...
that NK1 receptor-expressing neurons are critical for the development of injury-induced hypersensitivity and are required for the development of central sensitization (Khasabov et al. 2002; Mantyh et al. 1997; Nichols et al. 1999). A recent report has shown that the NK1 receptor is only expressed in lamina I–III neurons (NeuN-positive) from the adult rat spinal cord (Khan et al. 2008). However, in our dorsal horn cultures, the percentage of neurons expressing NK1 is much higher than 34%, since 85% of these neurons are Kv4.2 positive, and 86% of Kv4.2-positive neurons are NK1 positive. The difference could be due to a species, developmental-stage issue or altered protein expression associated with culturing.

PKCγ has also been strongly implicated in the development of injury-induced, persistent pain conditions, particularly in the development of tactile allodynia (Braz and Basbaum 2009). PKCγ neurons have been shown to be located in lamina II (Malmberg et al. 1997). Our result from cultured dorsal horn neurons showed that 85% of Kv4.2-expressing neurons are PKCγ positive, whereas 100% of PKCγ neurons are immunoreactive for Kv4.2. Confocal imaging showed that PKCγ immunoreactivity overlaps with Kv4.2 immunoreactivity in the spinal cord dorsal horn. We have previously shown that activation of PKC by PMA reduces Kv4.2-mediated A-type currents and neuronal excitability in dorsal horn neurons via activation of ERK (Hu and Gereau 2003). The findings presented here are consistent with the hypothesis that PMA-induced inhibition of A-type currents in some dorsal horn neurons is likely mediated by activation of PKCγ.

It should be noted that our staining results revealed that NK-1 and PKCγ appear to be highly coexpressed in the cultured dorsal horn neurons. Previous reports from Todd’s lab (Polgar et al. 1999) showed that only 22% and 36.7% of the NK1 receptor-immunoreactive neurons are PKCγ immunoreactive in lamina I and III, respectively, from the adult rat spinal cord. However, the difference could be due to a species, developmental-stage issue or altered protein expression associated with culturing.

The electrophysiological results demonstrate that Kv4.2 is expressed in the soma, as we recorded somatic Kv4.2-mediated currents from cultured neurons without or with short processes, which is consistent with Kv4.2 somatic staining. Our confocal imaging data showed that VGT1/2 labeled both the soma and processes and highly colocalized with Kv4.2 in our culture conditions. However, previous reports suggest that VGT1/2 staining is restricted to dorsal horn synaptic terminals in fixed spinal cord sections, with little or no labeling associated with

Fig. 7. Colocalization of Kv4.2 and vesicular glutamate transporters (VGTs) in dorsal horn neurons. A: representative confocal images of Kv4.2 (red) and VGT1 or VGT2 (green) coimmunofluorescence in cultured dorsal horn neurons. B: representative confocal images of Kv4.2 (red) and VGT2 (green) coimmunofluorescence in the mouse dorsal horn. Merge of Kv4.2 and VGTs is shown in yellow. Original scale bars: A, 40 μm; B, 200 μm.
neuronal somata (Landry et al. 2004; Li et al. 2003; Todd et al. 2003). The relatively greater abundance of VGT1/2 labeling in the soma of cultured neurons may reflect altered protein expression or trafficking associated with the isolation of the cells, or this could be related to differences in the age of the animals used in preparing cultures vs. slices.

Previous studies have shown that GAD67-expressing neurons represent more than two-thirds of GABAergic inhibitory neurons in the superficial dorsal horn (Dougherty et al. 2005, 2009). Our present results reveal that these neurons express little or no Kv4.2-mediated A-type currents and that the Kv4.2 subunit is mainly expressed in excitatory neurons, indicating that inhibition of Kv4.2 activity likely increases excitation of predominantly excitatory neurons in the spinal dorsal horn circuit. These data are consistent with the hypothesis that mGlu5 modulation of Kv4.2-containing K⁺ channels occurs only in excitatory neurons in the spinal cord dorsal horn, suggesting that the pronociceptive actions of mGlu5/ERK signaling, which is mediated largely by downstream inhibition of Kv4.2 (Hu et al. 2006, 2007), are mediated by modulation of excitatory dorsal horn neurons.

It is important to point out that the lack of GFP expression does not indicate that neurons are not GABAergic. By focusing on the GAD67-GFP neurons, we may be restricting our anal-

Fig. 8. Colocalization of Kv4.2 and neurokinin 1 (NK1) or PKCγ. A: representative confocal images of Kv4.2 (red) and NK1 (green) immunofluorescence in dorsal horn neurons and in the mouse dorsal horn. B: representative confocal images of Kv4.2 (red) and PKCγ (green) immunofluorescence in dorsal horn neurons and in the mouse dorsal horn. Merge of Kv4.2 with NK1 or PKCγ is shown in yellow. Original scale bars: cultured cells, 40 μm; slices, 200 μm.
ysis to only a subset of GABAergic neurons, and thus our findings cannot be attributed to all GABAergic neurons in the dorsal horn. The relative abundance of A-type currents and firing patterns in this GFP-positive population of interneurons is therefore only a subset of GABAergic interneurons, and the abundance of these properties and of mGlu5-dependent modulation of these properties may differ in other GABAergic interneurons. It should also be noted that a recent paper from Andrew Todd’s group (Yasaka et al. 2010) indicates that there is a similar association of firing patterns indicative of A-type currents with excitatory neurons in lamina II from the adult rat. This study used immunohistochemical identification of GABAergic interneurons and did not rely on GFP expression from the GAD67 locus. Therefore, these findings in another species using different methods lend support to our overall interpretation.

ACKNOWLEDGMENTS

The authors thank Dr. Robert H. Edwards for providing the rabbit polyclonal antisera against GABA, and Dr. Robert H. Edwards for providing the rabbit polyclonal antivesicular glutamate transporters 1 and 2.

GRANTS

This work was supported by a grant from the National Institute of Neurological Disorders and Stroke (NS48602) to R. W. Gereau IV.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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