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Expression of A-type K^+ channel α subunits Kv4.2 and Kv4.3 in rat spinal lamina II excitatory interneurons and colocalization with pain-modulating molecules

Hsin-Yi Huang,[1,*] Jen-Kun Cheng,[2,*] Yang-Hsin Shih,[3] Pei-Hsuan Chen,[1] Chin-Lin Wang[1] and Meei-Ling Tsaur[1]
[1]Institute of Neuroscience, National Yang-Ming University, Brain Research Centre, University System of Taiwan, Taipei, Taiwan 112, Republic of China
[2]Department of Anaesthesiology, Mackay Memorial Hospital, Taipei, Taiwan 251, Republic of China
[3]Division of Neurosurgery, Neurological Institute, Veterans General Hospital, Taipei, 112, Taiwan, Republic of China

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Abstract

Voltage-gated K^+ channel α subunits Kv4.2 and Kv4.3 are the major contributors of somatodendritic A-type K^+ currents in many CNS neurons. A recent hypothesis suggests that Kv4 subunits may be involved in pain modulation in dorsal horn neurons. However, whether Kv4 subunits are expressed in dorsal horn neurons remains unknown. Using immunohistochemistry, we found that Kv4.2 and Kv4.3 immunoreactivity was concentrated in the superficial dorsal horn, mainly in lamina II. Both Kv4.2 and Kv4.3 appeared on many rostrocaudally orientated dendrites, whereas Kv4.3 could be also detected from certain neuronal somata. Kv4.3(+) neurons were a subset of excitatory inerneurons with calretinin(+)/calbindin(–)/PKCγ(–) markers, and a fraction of them expressed μ-opioid receptors. Kv4.3(+) neurons also expressed ERK2 and mGluR5, which are molecules related to the induction of central sensitization, a mechanism mediating nociceptive plasticity. Together with the expression of Kv4.3 in VR1(+) DRG neurons, our data suggest that Kv4 subunits could be involved in pain modulation.

Introduction

Nociceptive primary sensory neurons, the cell bodies of which reside in dorsal root ganglia (DRG), receive pain signals from peripheral nerve endings and transduce it through their afferents to the dorsal horn of the spinal cord. In the dorsal horn, DRG afferents innervate not only the secondary sensory neurons but also the interneurons, which are located mainly in lamina II. Lamina II interneurons can produce either excitatory or inhibitory effects to the secondary sensory neurons such that pain signals can be modulated (Parent, 1996; Altman & Bayer, 2001; Bear *et al*., 2001). Molecules related to pain modulation are expressed in lamina II, such as μ-opioid receptors (MOR1; Arvidsson *et al*., 1995; Kemp *et al*., 1996), metabotropic glutamate receptor 5 (mGluR5; Alvarez *et al*., 2000; Karim *et al*., 2001), adenosine receptor 1 (A_1R) (Schulte *et al*., 2003), and external signal-regulated kinase (ERK; Flood *et al*., 1998; Ji *et al*., 1999).

A-type K^+ currents (I_As), activated transiently and inactivated rapidly, are key components in the control of neuronal excitability (Hille, 2001). I_As have been detected from the somatodendritic domain of ~10% of lamina II neurons (Grudt & Perl, 2002). At the behavioural level, ERK is activated following nociceptive afferent input, and inhibition of ERK activation can suppresses pain responses (Ji *et al*., 1999). At the cellular level, inhibition of ERK activation can increase I_As in cultured superficial dorsal horn neurons, suggesting that there is basal ERK activity that tonically inhibits I_As in lamina II neurons (Hu *et al*., 2003). Kv4 subunits, which are voltage-gated K^+ (Kv) channel α subunits, can evoke I_As in heterologous expression systems (Coetzee *et al*., 1999). Interestingly, Kv4 subunits are effective ERK substrates *in vitro* and *in vivo* (Adams *et al*., 2000). Thus, it has been hypothesized that regulation of Kv4 channel activity by ERK in dorsal horn neurons may underlie the central sensitization, a fundamental cellular mechanism of chronic pain (reviewed in Ji *et al*., 2003).

Among the Kv4 subunits (Kv4.1, Kv4.2, and Kv4.3), Kv4.2 and Kv4.3 have been detected from many neurons in the central nervous system (CNS), at both mRNA and protein levels. In addition, Kv4.2 and Kv4.3 are the major components of I_As in the somatodendritic domain of most CNS neurons examined (reviewed in Birnbaum *et al*., 2004; Rhodes *et al*., 2004). In contrast, Kv4.1 mRNAs are at a much lower level compared with Kv4.2 or Kv4.3 mRNAs in the CNS (Serodio & Rudy, 1998). So far, no study using anti-Kv4.1 antibody has been reported. Therefore, it is likely that Kv4.2 and/or Kv4.3 also underlie the I_As detected from the somatodendritic domain of lamina II neurons. However, whether Kv4.2 and/or Kv4.3 are expressed in lamina II neurons remain unknown. In this report, we examined the distribution of Kv4.2 and Kv4.3 in rat spinal cord and DRG, and also investigated whether they are colocalized with molecules related to pain modulation.

Correspondence: Dr Meei-Ling Tsaur, as above.
E-mail: mltsaur@ym.edu.tw

*H.-Y. H. and J.-K. C. contributed equally to this work.

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Materials and methods

Animals

Adult male Sprague–Dawley rats (250–300 g) were provided by the Animal Centre, National Yang-Ming University. National guidelines

of animal care were followed, and all the experiments were approved by the local ethics committee of the National Yang-Ming University.

Specificity of Kv4.2 and Kv4.3 antibodies

Kv4.2 and Kv4.3 polyclonal antibodies purified from rabbit sera, as well as Kv4.2 immunogenic peptides (corresponding to residues 454–469 of rat Kv4.2), were purchased from Alomone Laboratories (Jerusalem, Israel). The specificity of Kv4.3 antibody has been confirmed previously (Hsu *et al*., 2003). The specificity of Kv4.2 antibody has been examined by immunoprecipitation using Kv4.2 and Kv4.3 proteins synthesized by *in vitro* transcription/translation, and Kv4.2 antibody did not cross-react with Kv4.3 proteins (Tsaur *et al*., 2001). The specificity of Kv4.2 antibody has been also examined by immunohistochemistry in adult rat brain, and the characteristic pattern could be abolished completely by preincubation with excess Kv4.2 immunogenic peptides (Tsaur *et al*., 2001). Here, to confirm the specificity of Kv4.2 antibody, Western blot was performed using the method described below.

Membrane protein preparation and Western blot

Adult rats were anaesthetized with sodium pentobarbital (120 mg/kg) and decapitated. The brains were removed, chopped into small pieces, and homogenized in ice-cold sucrose buffer (0.32 M sucrose, 1 mM EDTA, 50 mM Tris pH 7.4) containing 1 μg/mL pepstatin, leupeptin, and aprotinin, 0.2 mM 4-(2-aminoehtyl)-benzenesulphonyl fluoride, 0.1 mg/mL benzamidine, and 8 μg/mL calpain inhibitors I and II (Sigma, St. Louis, MO, USA). After centrifugation at 2000 ***g*** for 10 min at 4 °C to remove debris, the supernatant was centrifuged at 100 000 ***g*** for 1 h at 4 °C. The pellet was suspended in sucrose buffer and aliquots were stored at −80 °C. Protein concentration was determined by the Bradford method (Harlow & Lane, 1999). Approximately 25 μg membrane proteins were solubilized in sodium dodecyl sulphate (SDS) sample buffer containing 5% 2-mercaptoethanol, then separated by 7.5% SDS/polyacrylamide gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. After a brief rinse in phosphate-buffered saline (PBS), nitrocellulose membranes were blocked for nonspecific binding with 5% nonfat milk in PBSW (PBS containing 0.05% Tween 20) for 1 h, washed in PBSW, and incubated with rabbit anti-Kv4.2 primary antibody at 0.3 μg/mL in PBSW containing 1% bovine serum albumin (BSA) for another hour, with or without preabsorption with 1 μg/mL Kv4.2 immunogenic peptides. After washing, goat anti-rabbit secondary antibody conjugated with horseradish peroxidase at 1 : 10000 (Amersham, Arlington Heights, IL, USA) was applied for 45 min. In the presence of chemiluminescent reagents (NEN, Boston, MA, USA), immunoreactive bands were visualized by exposing the nitrocellulose membranes to X-ray film. All steps were performed at room temperature.

Perfusion

Rats were injected intraperitoneally with 4000 U heparin/kg body weight to prevent blood clotting. After 5 min, animals were anaesthetized by another injection of sodium pentobarbital (120 mg/kg), and perfused transcardially with normal saline followed by 2% paraformaldehyde plus 0.25% glutaldehyde in PBS. The spinal cord and DRG at the cervical and thoracic segments were removed and post-fixed in the same fixative at room temperature for 2 h. All specimens were cryoprotected in 30% (w/v) sucrose.

Single antigen immunohistochemistry

Spinal cords were cut transversely or parasagittally with a cryostat into 20 or 30-μm floating sections. The 20 or 30-μm sections of DRG were mounted directly onto gelatin-coated slides in a random orientation. After washing in PBS, followed by PBS containing 0.3% Triton X-100 (PBST) 2 ×10 min, sections were treated with 0.3% hydrogen peroxide in PBST for at least 15 min to exhaust the endogenous hydrogen peroxidase. Nonspecific binding was blocked by 3% normal goat serum plus 2% BSA in PBST for 1 h for floating sections or 2 h for sections on slides. Sections were incubated at room temperature overnight with primary antibody in PBST plus 3% normal goat serum. The primary rabbit polyclonal antibodies and the concentrations applied to floating sections were anti-Kv4.2 (1 μg/mL) and anti-Kv4.3 (1.5 μg/mL), respectively. Concentrations of primary antibodies were doubled for sections on slides. Sections were washed three times for 10 min each with PBST, and incubated with goat anti-rabbit biotinylated secondary antibody (1 : 1000; Pierce, Rockford, IL, USA) for 1.5 h at room temperature. After washing three times with PBST, avidin-biotin-horseradish peroxidase complex (ABC complex; Pierce) in PBST was applied in a 1 : 160 dilution to floating sections for 1 h, or in a 1 : 80 dilution to sections on slides for 1.5 h. Antigens were visualized by combining equal volumes of an ammonium nickel sulphate solution (30 mg/mL in 0.1 M sodium acetate, pH 6.0) and a diaminobenzidine solution (4 mg/mL in PBS) in the presence of 0.01% hydrogen peroxide. Floating sections were spread flat on slides, air dried, rinsed with distilled water for 1 min, and dehydrated through an ethanol gradient (70% once, 95% twice, and 100% twice) for 1.5 min each and then in xylene for 3 min twice. Sections were coverslipped using Permount mounting medium (Merck, Darmstadt, Germany). Images were acquired with a Nikon DMX1200 connected digital camera to a Nikon Eclipse E800 light microscope (Nikon, Melville, NY, USA), and processed with Adobe Photoshop 8.0 software (Adobe, Mountain View, CA, USA).

Double antigen immunofluorescent staining

For double staining with two antibodies derived from different species, the following procedure was used. Sections were processed similarly to that described under 'Single antigen immunohistochemistry', except that treatment with hydrogen peroxide was omitted. Sections were incubated simultaneously with anti-Kv4.2 (2 μg/mL) or anti-Kv4.3 (3 μg/mL) antibody with one of the following primary antibodies. Mouse monoclonal antibodies included anti-calretinin (1 : 100; Chemicon, Temecula, CA, USA), anti-ERK2 (1 : 500; Santa Cruz, Santa Cruz, CA, USA), anti-MAP2 (1 : 100; Sigma, St. Louis, MO, USA), anti-parvalbumin (1 : 1000; Sigma), anti-PKCγ (1 : 200; BD Transduction Laboratories, San Diego, CA, USA), and anti-synaptophysin (1 : 500; Santa Cruz). Goat antibodies anti-calbindin (1 : 100; Santa Cruz) and anti-VR1 (1 : 100; Santa Cruz) were also used. Secondary antibodies included Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1 : 200; Molecular Probes, Eugene, OR, USA), Alexa Fluor 594-conjugated donkey antimouse IgG (1 : 500; Molecular Probes), or rhodamine Red-X-conjugated donkey anti-goat IgG (1 : 400; Jackson ImmunoResearch, West Grove, PA, USA). Sections were spread flat on slides, air dried, and mounted with antifading medium Fluoromount-G (Southern Biotech, Birmingham, AL, USA) under coverslips. Images were collected using Olympus FV300 confocal laser scanning microscope and processed with Adobe Photoshop 8.0 software.

For double staining using both antibodies derived from the same species (rabbit in this report), we followed the tyramide amplification

method (Shindler & Roth, 1996). Briefly, on the first day, the first primary antibody was applied at a concentration much lower than normally used for double immunofluorescent staining. On the second day, with intervening washes with PBST, the signal for the first primary antibody was amplified sequentially by the biotinylated donkey anti-rabbit IgG (1 : 1000; Jackson ImmunoResearch), ABC complex (1 : 200), biotinylated tyramide (1 : 1000), and rhodamine Red-X-conjugated streptavidin (1 : 200; Jackson ImmunoResearch). The second primary antibody was then applied overnight at a concentration normally used for double immunofluorescent staining. On the third day, Alexa Fluor 488 conjugated donkey antirabbit IgG (1 : 200) was applied to visualize the second primary antibody. Anti-Kv4.3 and anti-MOR1 (kindly provided by Dr Robert Elde, University of Minnesota) were used as the first primary antibodies, respectively. To minimize the nonspecific signal amplified by tyramide, negative controls were performed as follows. First, to obtain enough positive red signal derived from the first primary antibody and to minimize background green signal derived from the second secondary antibody, selected dilutions of anti-Kv4.3 (0.3 μg/mL) and anti-MOR1 (1 : 25000) were chosen, in the absence of the second primary antibody (Figs 4E and 5A). Then, we tested the specificity of tyramide amplification by applying a second primary antibody with a known nonoverlapping pattern, such as anti-A_1R (1 : 100; Affinity Bioreagent, Golden, CO, USA). A_1R-IR (in certain IIi neuronal soma) did not overlap with MOR1-IR (Schulte *et al*., 2003) or Kv4.3-IR (data not shown). For Kv4.3/Kv4.2 double labelling, sections were first processed for anti-Kv4.3 (0.3 μg/mL) and then anti-Kv4.2 (1.5 μg/mL). For Kv4.3/mGluR5, sections were processed for anti-Kv4.3 (0.3 μg/mL) and then anti-mGluR5 (1 : 500; Upstate, Charlottesville, VA, USA). For MOR1/Kv4.2 or MOR1/Kv4.3, the sections were processed for anti-MOR1 (1 : 25 000) and then anti-Kv4.2 (1.5 μg/mL) or anti-Kv4.3 (3 μg/mL).

Quantitative measurements

Lamina I, outer (IIo), and inner (IIi) lamina II appeared as distinctive bands in dark field illumination due to the relative absence of myelinated fibres. Dimensions of the Kv4.3(+) somata were measured in 30-μm spinal cord slices in the parasagittal orientation and labelled with fluorescence. The largest dimension of a fluorescence-labelled cell could be visualized by changing the Z-axis of confocal microscope. The quantitative measurements of cell bodies expressing Kv4.3 and other neuronal markers were carried out by counting single and double-labelled cells. Then, the labelled cells from the sampled regions were pooled for statistical analysis. Values shown are the mean ± SEM. The number in parentheses after *n*-value (numbers of experiments) gives the number of total cells counted. All data represent the cell counts from three adult rats.

Results

Western blot of Kv4.2 antibody

Using rat brain membrane proteins for Western blot analysis, we found that Kv4.2 antibody recognized a major protein band at ~72 kDa, which is consistent with the molecular weight of Kv4.2 protein (Fig. 1, left lane). When Kv4.2 antibody was preabsorbed with excess Kv4.2 immunogenic peptides, the 72 kDa band was no longer detected (Fig. 1, right lane). A minor band with molecular weight slightly > 72 kDa was also detected, which could be also competed away in the presence of excess K4.2 immunogenic peptides (Fig. 1). Because Kv4.2 can be phosphorylated by ERK2 and CaMKII *in vitro* and *in vivo* (Adams *et al*., 2000; Varga *et al*., 2004), it is likely that the minor band represents the phosphorylated form of Kv4.2 proteins. Thus, Western blot, together with our previous analysis using immunohistochemistry and immunoprecipitation (Tsaur *et al*., 2001), confirmed the specificity of the Kv4.2 antibody.

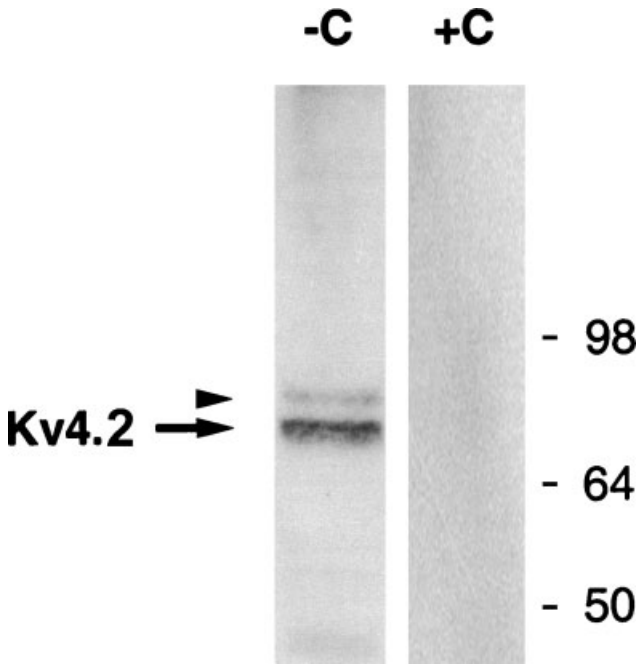

FIG. 1. Characterization of Kv4.2 antibody by Western blot. Crude membrane proteins isolated from adult rat brain were separated by 7.5% SDS/PAGE, transferred to nitrocellulose membrane, and incubated with anti-Kv4.2 antibody, in the absence (–C) or presence (+C) of Kv4.2 immunogenic peptides as competitor. Left lane, the major band (arrow) at position of ~72 kDa is consistent with the molecular weight of Kv4.2 protein. The minor band (arrowhead) is likely to be the phosphorylated form of Kv4.2 protein. Right lane, both bands are undetectable in the presence of excess competitor peptides. The positions of molecular mass standards (in kDa) are shown.

Kv4.2 is present on the dendrites of certain lamina II neurons

In the transverse orientation of adult rat spinal cord, Kv4.2-immunoreactivity (Kv4.2-IR) was observed mainly in lamina II (Fig. 2A and B). Lamina II can be divided into the outer (IIo) and inner (IIi) regions. Because the ratio of thicknesses between the IIo and IIi is approximately 1 : 3, we further divided the IIi into dorsal, medial and ventral parts. The highest intensity of Kv4.2-IR was detected in a zone located in the dorsal part of IIi (Fig. 2B), where Kv4.2(+) dot-like structures were more concentrated than the other parts of lamina II (Fig. 2C). Weak Kv4.2-IR could be also detected in laminae I and III. In the parasagittal orientation through lamina II, Kv4.2-IR was found in long fibrous structures extending rostro-caudally (Fig. 2D–F). No somatic staining could be detected from either orientation (Fig. 2C and F). Using double immunofluorescent staining, we found that all fibrous Kv4.2-IR was colocalized with MAP2-IR (a dendritic marker); however, only part of MAP2-IR overlapped with Kv4.2-IR (Fig. 4A). Thus, Kv4.2 is expressed on the dendrites of certain lamina II neurons.

Kv4.3 appears on the somata and dendrites of certain lamina II neurons

From the transverse view of spinal cord, similar to Kv4.2-IR, strong Kv4.3-IR was also found in lamina II (Fig. 3A). Kv4.3-IR was

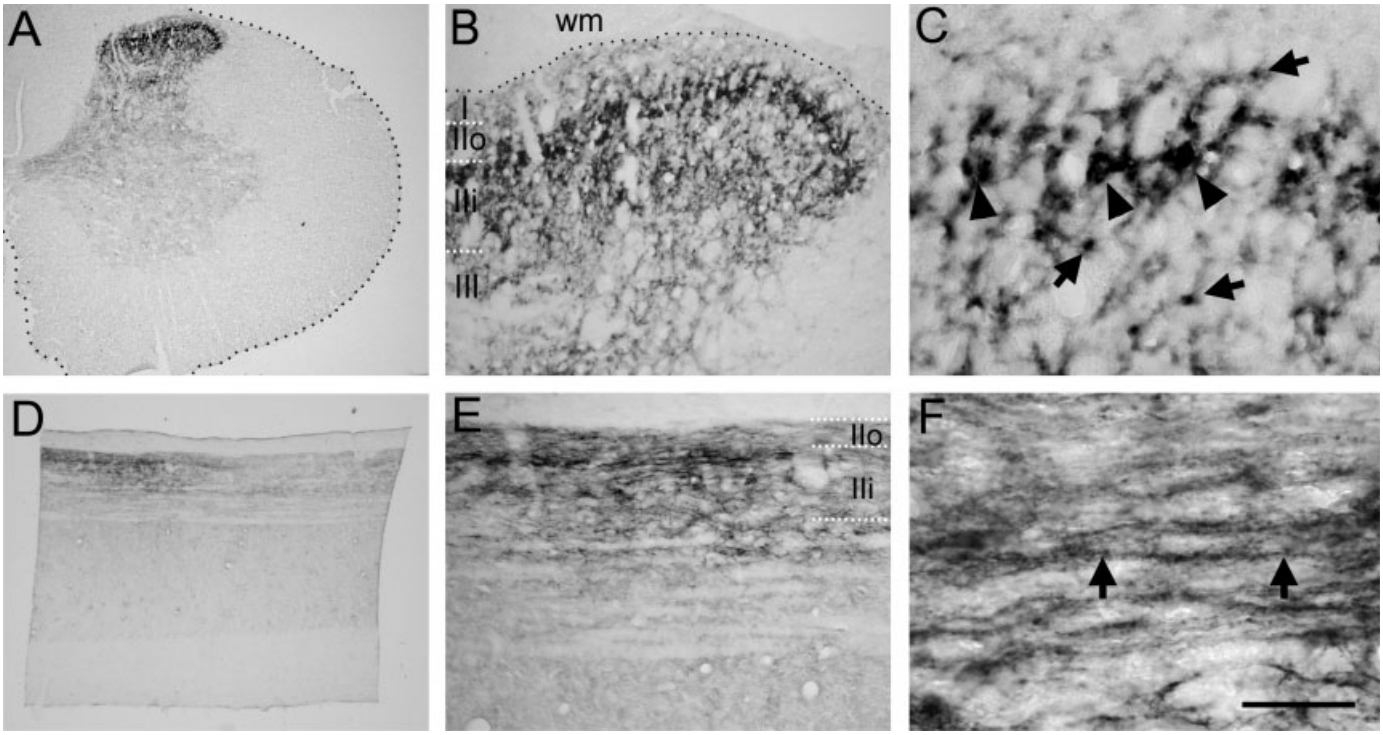

FIG. 2. Expression of Kv4.2 in spinal cord. Transverse (A–C) and parasagittal (D–F) sections of adult rat spinal cord at the cervical level were immunostained with Kv4.2 antibody. (A) Kv4.2-IR is apparent in dorsal horn. (B) Higher magnification of the dorsal horn shown in A. Kv4.2-IR is distributed mainly in lamina II, with the strongest intensity in the dorsal part of IIi. (C) Higher magnification of lamina II shown in B. Kv4.2-IR appears in dot-like structures, either concentrated in the dorsal part of IIi (arrowheads) or sparsely distributed (arrows). (D) Kv4.2-IR in dorsal horn (the left side) is shown from a parasagittal view of spinal cord. (E) Higher magnification of the dorsal horn shown in D. Kv4.2-IR is obvious in lamina II. (F) Higher magnification of the fibrous staining shown in E. Kv4.2(+) processes (arrowheads) extend mainly in a rostro-caudal orientation. No somatic staining can be seen in either orientation. Scale bars, 5.4 mm (A); 120 μm (B); 38 μm (C); 7.5 mm (D); 200 μm (E); 33 μm (F).

distributed homogeneously in lamina II, except the ventral part of IIi (Fig. 3B). Under higher magnification, Kv4.3-IR was detected in many dot-like structures, similar to Kv4.2 (Fig. 3C). Interestingly, Kv4.3-IR also appeared in some longitudinally oval structures, which look like cell bodies (the insert in Fig. 3C, and the middle panel of Fig. 5C). From the parasagittal view, Kv4.3-IR was obvious on certain somatic surfaces and their processes in the IIo and IIi (Fig. 3D–F). Kv4.3(+) processes extended in the rostro-caudal direction (Fig. 3E and F), similar to Kv4.2(+) dendrites. In a measurement using parasagittal sections, the average diameter of Kv4.3(+) somata was 21.8 ± 3.2 μm rostro-caudally and 9.2 ± 2.0 μm dorso-ventrally ($n = 3$; 35 cells). Almost all Kv4.3-IR was colocalized with MAP2-IR in the somatic membranes and their processes, however, only part of MAP2-IR overlapped with Kv4.3-IR (Fig. 4B). These results indicate that Kv4.3 is expressed on the somatic surfaces and dendrites of certain lamina II neurons.

Kv4.3 is expressed in the somata of nociceptive DRG neurons

Anterograde analysis via DRG afferents reveals the IIo receives terminations from the most finest unmyelinated fibres (C-fibres), while the IIi receives endings from some C-fibres and thinly myelinated fibres (Aδ fibres; Parent, 1996). We have observed Kv4.2 and Kv4.3 in the somatodendritic domain of certain lamina II neurons. To check whether Kv4.2 and Kv4.3 were also present in DRG afferents and their terminals distributed in the dorsal horn, double staining with Tau1 (a marker for axons) or synaptophysin (a marker for axon terminals) was performed in both spinal cord and DRG. Kv4.2- and Kv4.3-IR showed no overlap with Tau1- or synaptophysin-IR (data not shown), suggesting that Kv4.2 and Kv4.3 are probably not present in axons and/or nerve terminals. Kv4.2 was also absent from the somatodendritic domain of DRG neurons. Interestingly, we found Kv4.3-IR in the somata of small-diameter DRG neurons (Fig. 3G). The average somatic diameter of Kv4.3(+) DRG neurons was 27.3 ± 3.2 μm ($n = 3$; 60 cells). Vallinoid receptor 1 (VR1) and parvalbumin are markers for the nociceptive and proprioceptive DRG neurons, respectively. Colocalization with VR1 indicates that Kv4.3 is expressed in the nociceptive DRG neurons (Fig. 4C). The average size of parvalbumin(+) DRG neurons are larger than that of Kv4.3(+) DRG neurons, and none of the Kv4.3(+) DRG neurons expressed parvalbumin (Fig. 4D). Interestingly, in the nociceptive DRG neurons, Kv4.3-IR appeared in the cytoplasm instead of somatic surface (Figs 3G, and 4C and D). In summary, Kv4.3 is expressed in the somatic cytoplasm of nociceptive DRG neurons.

Kv4.3(+) lamina II neurons also express Kv4.2

Both Kv4.2 and Kv4.3 were present on the dendrites of certain lamina II neurons. To examine whether they were colocalized, double staining with Kv4.3 signal amplified by tyramide was performed. In the absence of Kv4.2 antibody, Kv4.3-IR in dorsal horn was similar to the pattern shown in Fig. 3A (Fig. 4E, left panel), and nonspecific staining contributed by the second secondary antibody was not detected (Fig. 4E, middle panel). In the presence of Kv4.2 antibody, Kv4.2-IR and Kv4.3-IR were colocalized in lamina II (Fig. 4F). Under higher magnification, almost all Kv4.3(+) dendrites showed Kv4.2-IR,

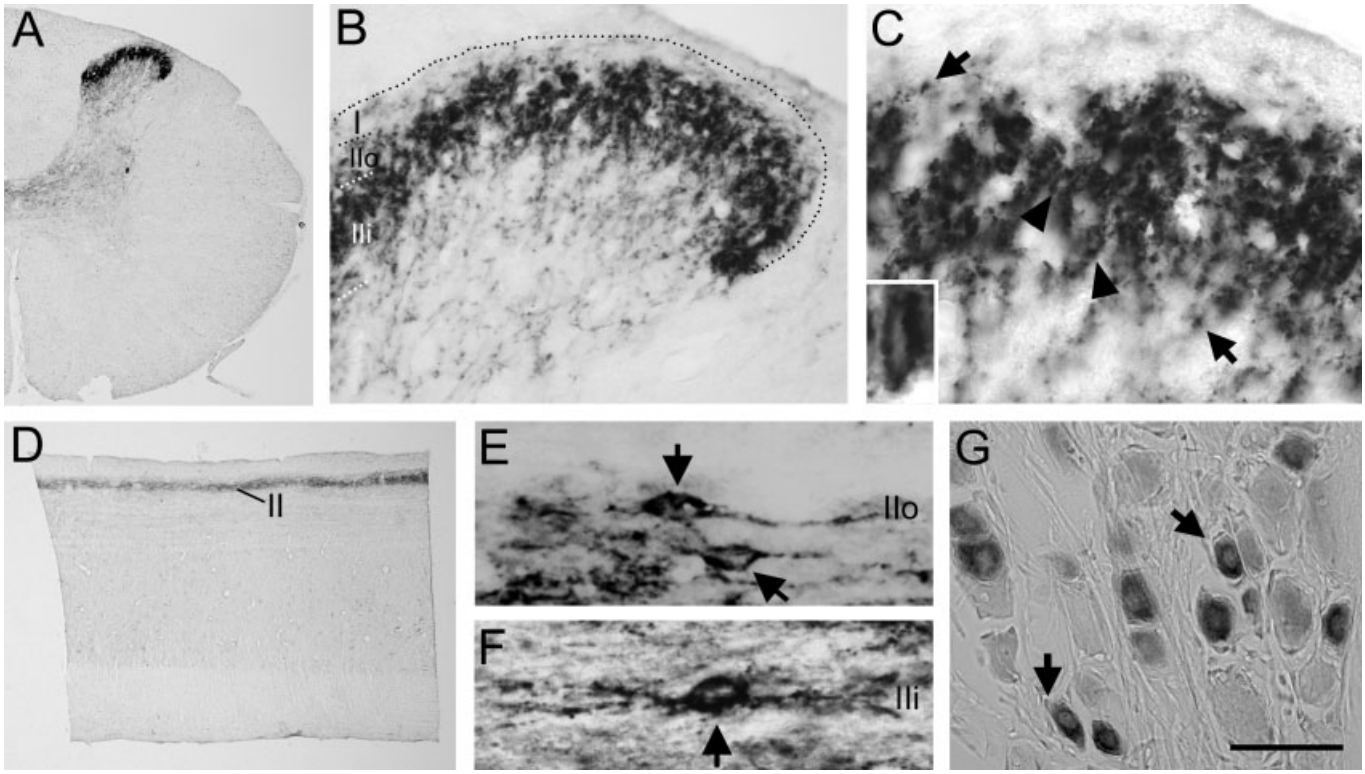

FIG. 3. Localization of Kv4.3 in spinal cord and DRG. Transverse (A–C) and parasagittal (D–F) sections of spinal cord at the thoracic level, as well as a random orientation of DRG (G), were immunostained with Kv4.3 antibody. (A) Kv4.3-IR is concentrated in the superficial dorsal horn. (B) Kv4.3-IR is distributed evenly throughout lamina II, except the ventral one-third of IIi. (C) Higher magnification of lamina II shown in B. Kv4.3-IR is present in dot-like structures (arrows), either concentrated (arrowheads) or sparsely distributed (arrows), similar to Kv4.2. Kv4.3-IR also appears in some cell body-like structures in lamina II (insert). (D) Kv4.3-IR in the superficial spinal cord is shown from a parasagittal view. (E, F) Kv4.3(+) neurons in the IIo and IIi are shown, respectively. (G) Kv4.3(+) DRG neurons are smaller in diameter than Kv4.3(–) DRG neurons. Scale bars, 4.6 mm (A); 110 μm (B); 36 μm (C; 18 μm for insert); 7.0 mm (D); 28 μm (E); 30 μm (F); 67 μm (G).

however, some Kv4.2(+) dendrites did not show Kv4.3-IR (Fig. 4G). These results indicate that Kv4.3(+) lamina II neurons also express Kv4.2. With somatic staining, it was easier to identify the cell type of Kv4.3(+) lamina II neurons.

Kv4.3 is expressed in a subset of excitatory interneurons in lamina II

Lamina II is composed mainly by inhibitory and excitatory interneurons, which are important in modulating pain signal transduction. Several calcium binding proteins have been used as markers to distinguish excitatory and inhibitory interneurons. In lamina II, parvalbumin is present in certain inhibitory interneurons, whereas calbindin(+) and calretinin(+) neurons represent the majority of excitatory interneurons, with a subpopulation expressing both calbindin and calretinin (Ren & Ruda, 1994; Albuquerque *et al*., 1999). To characterize Kv4.3(+) lamina II neurons, double staining with each of these calcium binding proteins was performed. We found that Kv4.3-IR was completely absent from parvalbumin(+) and calbindin(+) neurons (data not shown). Almost all Kv4.3(+) neurons were calretinin(+), but only ∼37% of calretinin(+) neurons ($n = 3$; 22 in 59 cells) showed Kv4.3-IR (Fig. 4H). Thus, Kv4.3 appeared in the calretinin(+)/calbindin(–) excitatory interneurons.

Protein kinase C (PKC)γ is a marker for another group of excitatory interneurons in lamina II (Polgar *et al*., 1999). Using double staining, we found that Kv4.3-IR was present in the IIo as well as the dorsal and medial parts of IIi, whereas PKCγ-IR was located mainly in the ventral part of IIi (data not shown). This non-overlapping indicates that Kv4.3 is absent from the PKCγ(+) excitatory interneurons. In summary, Kv4.3 is expressed in a subset of excitatory interneurons in lamina II, which are calretinin(+), calbindin(–), and PKCγ(–).

Kv4.2 and Kv4.3 are present in MOR1(+) neurons in lamina II

MOR1(+) lamina II neurons represent another group of excitatory interneurons, and MOR1 is expressed preferentially in their somatodendritic domain (Arvidsson *et al*., 1995; Kemp *et al*., 1996). MOR1(+) neuronal somata appeared in the dorsal part of IIi, which were calretinin(+)/calbindin(–)/PKCγ(–) (data not shown), similar to Kv4.3(+) neurons. Double staining was performed to examine whether MOR1(+) neurons also express Kv4.3. We found that MOR1-IR and Kv4.3-IR partially overlapped in a zone within lamina II (Fig. 5A and B). Under higher magnification, almost all MOR1(+) somata showed Kv4.3-IR; however, Kv4.3(+) somata in the IIo and the medial part of IIi did not show MOR1-IR (Fig. 5C). Double staining was also performed to check the colocalization of MOR1 and Kv4.2. We found that all MOR1(+) dendrites showed Kv4.2-IR, but only part of Kv4.2(+) dendrites were MOR1(+) (Fig. 5D). These results indicate that Kv4.2 and Kv4.3 are present in the somatodendritic domain of MOR1(+) excitatory interneurons situated in lamina II.

Adenosine receptor 1 (A_1R), another molecule related to pain modulation, is highly expressed in the somatodendrtic domain of a subset of lamina II neurons, different from MOR1 (Schulte *et al*.,

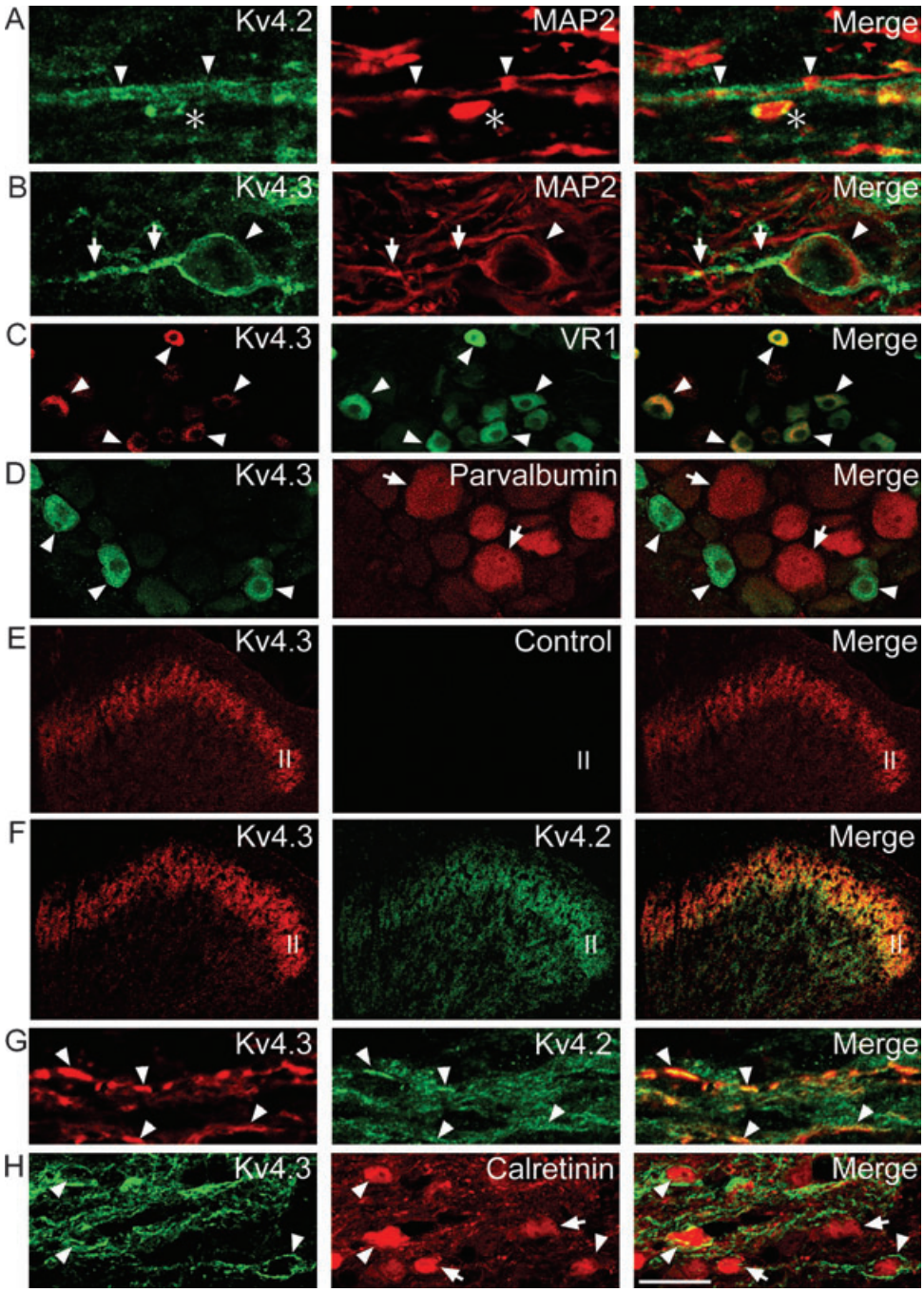

FIG. 4. Kv4.2 and Kv4.3 in lamina II excitatory interneurons as well as Kv4.3 in nociceptive DRG neurons. Parasagittal (A, B, G and H) and transverse (E and F) sections of spinal cord at the thoracic level, and a random orientation of DRG (C and D), were used for double immunofluorescent staining. (A) In lamina II, Kv4.2 (green) is colocalized with MAP2 (red) mainly in the rostro-caudally orientated dendrites (arrowheads). Less frequently, Kv4.2(+) dendrites in other orientations (asterisk, the transverse orientation of a dendrite) can be detected. (B) In some lamina II neurons, Kv4.3 (green) is coexpressed with MAP2 (red) in the somatic surfaces (arrowhead) and dendrites (arrows). (C) Kv4.3-IR (red) and VR1-IR (green) overlap in the small-diameter DRG neurons (arrowheads). (D) Kv4.3(+) DRG neurons (green, arrowheads) are smaller in diameter than parvalbumin(+) DRG neurons (red, arrows). (E) A control experiment of F. In the absence of Kv4.2 antibody, Kv4.3-IR amplified by tyramide (red) and a background control (green) in dorsal horn are shown. (F) In dorsal horn, Kv4.3-IR amplified by tyramide (red) and Kv4.2-IR (green) overlap in lamina II. (G) In lamina II, Kv4.2-IR (green) is present in both Kv4.3(+)(red, arrowheads) and Kv4.3(–) dendrites. (H) In lamina II, some calretinin(+) excitatory interneurons (red, in the cytoplasm) express Kv4.3 on somatic surfaces (green, arrowheads), but some don't (arrows). Scale bars, 7.7 μm (A); 20 μm (B); 63 μm (C); 54 μm (D); 110 μm (E and F); 22 μm (G); 13 (H).

2003). To test whether Kv4.3(+)/MOR1(–) neurons express A_1R, double staining was performed. No overlap was observed between Kv4.3-IR and A_1R-IR, although they were both distributed in the somatodendritic domain of some lamina II neurons (data not shown).

Kv4.3(+) lamina II neurons express both ERK2 and mGluR5

To test the possibility whether Kv4 subunits are the downstream effectors for the induction of central sensitization, a useful first step is

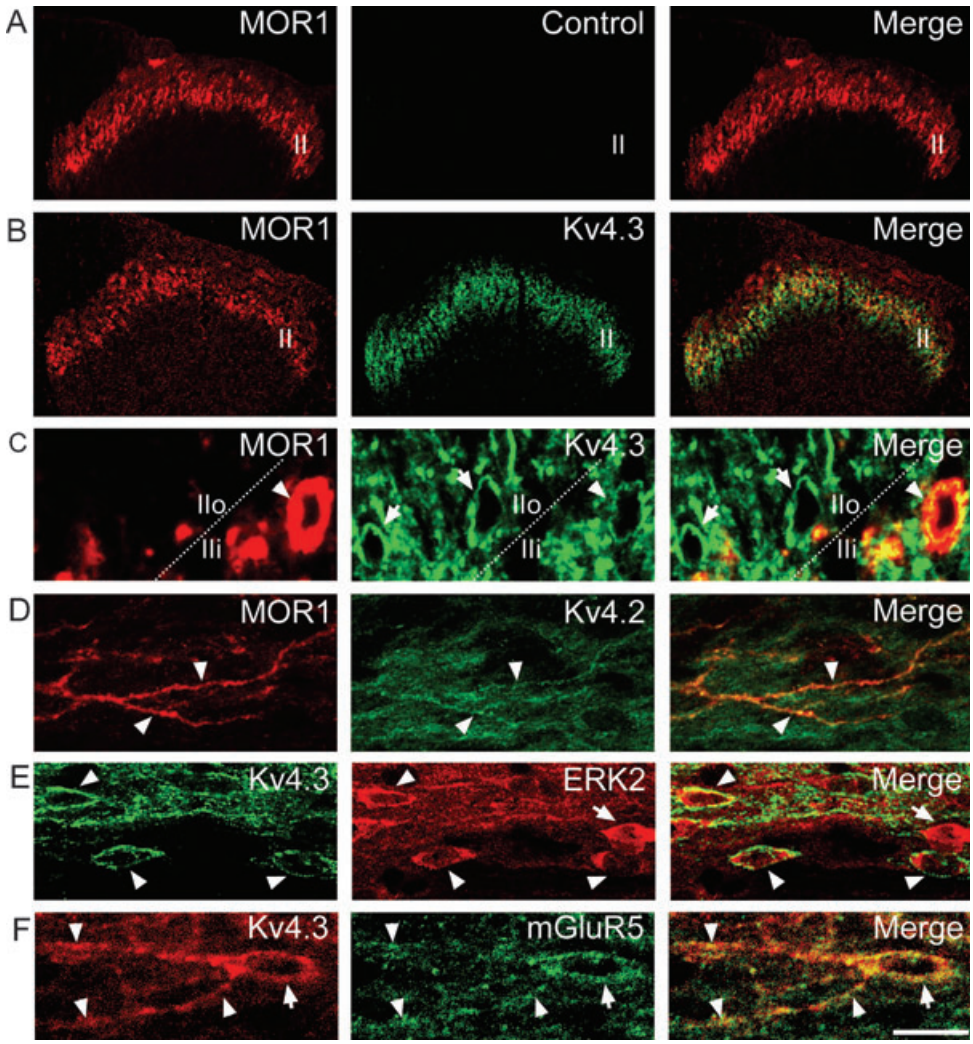

FIG. 5. Colocalization of Kv4.2 or Kv4.3 with pain-modulating molecules in lamina II excitatory interneurons. Transverse (A–C) or parasagittal (D–F) sections of spinal cord at the thoracic level were used for double immunofluorescent staining. (A) Control experiment of B. In the absence of Kv4.3 antibody, MOR1-IR amplified by tyramide (red) and a background control (green) are shown. (B) In dorsal horn, MOR1-IR (red) and Kv4.3-IR (green) overlap in a zone within lamina II. (C) In lamina II, Kv4.3(+) somata (green) in the dorsal part of IIi are MOR1(+) (red, arrowhead), whereasKv4.3(+) somata in the IIo are MOR1(–) (arrows). (D) In lamina II, Kv4.2-IR (green) appears in both MOR1(+) (red, arrowheads) and MOR1(–) dendrites. (E) In lamina II, some ERK2(+) neurons (red) express Kv4.3 on somatic surfaces (green, arrowheads), but some don't (arrows). (F) In lamina II, Kv4.3-IR (red) and mGluR5-IR (green) are colocalized on some neuronal surfaces (arrow) and their dendrites (arrowheads). Scale bars, 110 μm (A and B); 12 μm (C); 21 μm (D); 25 μm (E); 17 μm (F).

to map whether Kv4 channels are colocalized with the other molecules being implicated in this mechanism, such as ERK2 and mGluR5 (Ji *et al*., 2003). Because both ERK2-IR and mGluR5-IR are apparent in lamina II neuronal somata (Flood *et al*., 1998; Alvarez *et al*., 2000), double staining was performed to check whether they are colocalized with Kv4.3. In lamina II, we found that all Kv4.3(+) somata showed ERK2-IR in their cytoplasm (Fig. 5E). In addition, almost all Kv4.3-IR overlapped with mGluR5-IR in the somatic membranes and dendrites of certain lamina II neurons (Fig. 5F). Thus, these data suggest that Kv4 subunits may be involved in the central sensitization.

Discussion

We have demonstrated that Kv4.2 and Kv4.3 are not randomly distributed in spinal cord lamina II neurons, but are preferentially associated with a subpopulation of excitatory interneurons, which are calretinin(+)/calbindin(–)/PKCγ(–). These interneurons also express ERK2 and mGluR5, suggesting that Kv4.2 and Kv4.3 may be involved in the central sensitization. A fraction of them is MOR1(+), suggesting that Kv4.2 and Kv4.3 might also participate in the modulation of morphine analgesia. Furthermore, Kv4.3 is present in the VR1(+) DRG neurons, which are responsible for the perception of nociceptive signal.

Subcellular localization of Kv4.2 and Kv4.3

Kv4.2 and Kv4.3 have been detected from the somatodendritic domain of many neurons in the CNS. For example, Kv4.2 proteins are concentrated in the apical and basal dendrites of hippocampal and neocortical pyramidal neurons, and Kv4.3 proteins are present in the somata and dendrites of hippocampal, striatal and neocortical interneurons (Rhodes *et al*., 2004). Consistently, in spinal cord lamina II excitatory interneurons, we found Kv4.2 in the dendrites, Kv4.3 in the somata and dendrites, as well as the colocalization of Kv4.2 and Kv4.3 in the dendrites. We also observed Kv4.3 in the somatic domain of nociceptive DRG neurons. These data indicates that Kv4 channels

are present in the somatodendritic domain of neurons, not only in the central but also peripheral nervous system. However, without the examination by electron microscopy, the possibility that Kv4.2 and Kv4.3 are also present in the axon and/or nerve terminals has not been excluded.

At the subcellular level, the somatic Kv4.3-IR in the nociceptive DRG neurons appears obviously in the cytoplasm instead of cell surface, where K^+ channels normally exert their functions. Voltage-gated K^+ channels are composed of pore-forming and auxiliary subunits, and certain auxiliary subunits can promote the transport of pore-forming subunits from the cytoplasm to cell surface. Surface expression of Kv4 subunits can be greatly increased by Kvβ subunits, K^+ channel-interacting proteins (KChIPs), frequenin, K^+ channel accessory protein (KChAP), dipeptidyl aminopeptidase-like protein (DPPX), cytoskeletal protein filamin, and post-synaptic density-95 (PSD-95). In addition, trafficking of Kv4 subunits to plasma membrane can be modulated by post-translational modification, such as palmitoylation, glycosylation, and phosphorylation (reviewed in Birnbaum *et al*., 2004). In the nervous system, the subcellular localization of channel proteins is regulated by neuronal activity (Misonou *et al*., 2004). We speculate that, when certain neuronal activity (e.g. pain signals) is evoked in the nociceptive DRG neurons, it might activate certain auxiliary proteins or a post-translational modification mechanism, which promotes Kv4.3 transport from cytoplasm to cell surface. Subsequently, increase of Kv4 subunit density on plasma membrane will function as a shock absorber to protect neurons from damage by next strong input.

Kv4.2 and Kv4.3 in lamina II excitatory interneurons

Neurons situated in the middle region of lamina II were named by Ramon y Cajal (1909) as 'central cells'. The middle region of lamina II corresponds roughly to the dorsal part of IIi. It is known that central cell somata are located mainly in the IIi and some are in the IIo. In addition, central cells are excitatory interneurons, with moderately dense dendritic arbor extending mainly in the rostro-caudal direction of spinal cord (Grudt & Perl, 2002). Kv4.2 and Kv4.3 are abundantly expressed in certain excitatory interneurons in the IIo and IIi, especially Kv4.2 showing the highest expression in the dorsal part of IIi, similar to the location of central cells. Thus, from a morphological view, it is likely that Kv4.2 and Kv4.3 are expressed in the central cells.

Contribution of Kv4.2 and Kv4.3 to I_As

Grudt & Perl (2002) detected somatic I_As from two groups of lamina II neurons in hamster spinal cord. The first group is a subpopulation of central cells, the I_A-expressing central cells, which are ∼7.6% (11 in 144 cells) of all neurons in lamina II, with a rostro-caudal dimension of 17.33 ± 1.20 μm. The second group has been designated as medial-lateral neurons, ∼ 2.1% (3 in 144 cells) of lamina II neurons, with a rostro-caudal dimension of 22 μm. Medial-lateral neuron has a much larger dendritic span in both the medial-lateral and the dorso-ventral planes than central cells. However, due to limited number, the other characteristics remain unclear (Grudt & Perl, 2002). In the rat, the rostro-caudal dimension of Kv4.3(+) lamina II neurons is 21.8 ± 3.2 μm. Although the somatic size of Kv4.3(+) cells looks similar to that of medial-lateral neurons, the heavy Kv4.3 staining in lamina II implies that it is unlikely that Kv4.3 is expressed only in the medial-lateral neurons. As central cells and medial-lateral neurons intermingle within lamina II, we reason that Kv4.3 is expressed in both the medial-lateral neurons and the I_A-expressing central cells. Thus, combining the morphological and electrophysiological studies, it is likely that Kv4.2 and Kv4.3 are I_A contributors in the I_A-expressing central cells and medial-lateral neurons. So far five I_A contributors in the mammalian system have been reported, including Kv1.4, Kv3.4, Kv4.1, Kv4.2, and Kv4.3 (reviewed in Coetzee *et al*., 1999). In addition to Kv4.2 and Kv4.3, dense punctate Kv3.4-IR has been detected from the neuopil around neurons in laminae I–III, but no staining was apparent in the soma or dendritic membrane of these neurons (Brooke *et al*., 2004). Nevertheless, the possibility that Kv3.4 is another I_A contributor in the I_A-expressing central cells and/or medial-lateral neurons has not been excluded.

Safronov *et al*. (1996) recorded I_As from the cell body of small-diameter DRG neurons in the rat. In addition to Kv4.3, Kv1.4 has been detected from the somata of small-diameter DRG neurons (Rasband *et al*., 2001). Thus, both Kv1.4 and Kv4.3 are I_A contributors in the small-diameter DRG neurons.

Possible involvement of Kv4 subunits in pain modulation

It is known that four types of K^+ channels are involved in pain modulation, including inward-rectifier (such as K_{ATP} and GIRKs), calcium-activated, two-pore, and voltage-gated K^+ (Kv) channels. Among the Kv channel subfamilies, Kv7 (KCNQ) subfamily is currently being extensively investigated, because its openers (e.g. retigabine) are effective in several animal models of chronic pain. Besides, several reports indicate that Kv1 subfamily is related to pain modulation (reviewed in Ocana *et al*., 2004). Here, from a morphological view, at the level of spinal cord and DRG, we provide evidence that the Kv4 subfamily is probably involved in pain modulation. To test this possibility, animal models will be useful in future studies.

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Abbreviations

(+), Immunoreactive to a specific molecule as indicated; I, II, III, Rexed laminae of spinal cord are numbered in Roman numerals from dorsal to ventral; IIo, the outer region of lamina II; IIi, the inner region of lamina II; A_1R, adenosine receptor 1; CNS, central nervous system; DRG, dorsal root ganglion; ERK2, external signal-regulated kinase 2; I_As, A-type K^+ currents; IR, immunoreactivity; kDa, kilodaltons; Kv, voltage-gated K^+ channels; mGluR5, metabotropic glutamate receptor 5; MOR1, μ-opioid receptor; VR1, vanilloid receptor 1.

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