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Distinct cellular distributions of Kv4 pore-forming and auxiliary subunits in rat dorsal root ganglion neurons

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Abstract

Aims—Dorsal root ganglia contain heterogeneous populations of primary afferent neurons that transmit various sensory stimuli. This functional diversity may be correlated with differential expression of voltage-gated K⁺ (Kv) channels. Here, we examine cellular distributions of Kv4 pore-forming and ancillary subunits that are responsible for fast-inactivating A-type K⁺ current.

Main methods—Expression pattern of Kv α -subunit, β -subunit and auxiliary subunit was investigated using immunohistochemistry, *in situ* hybridization and RT-PCR technique.

Key findings—The two pore-forming subunits Kv4.1 and Kv4.3 show distinct cellular distributions: Kv4.3 is predominantly in small-sized C-fiber neurons, whereas Kv4.1 is seen in DRG neurons in various sizes. Furthermore, the two classes of Kv4 channel auxiliary subunits are also distributed in different-sized cells. KChIP3 is the only significantly expressed Ca²⁺-binding cytosolic ancillary subunit in DRGs and present in medium to large-sized neurons. The membrane-spanning auxiliary subunit DPP6 is seen in a large number of DRG neurons in various sizes, whereas DPP10 is restricted in small-sized neurons.

Significance—Distinct combinations of Kv4 pore-forming and auxiliary subunits may constitute A-type channels in DRG neurons with different physiological roles. Kv4.1 subunit, in combination with KChIP3 and/or DPP6, form A-type K⁺ channels in medium to large-sized A-fiber DRG neurons. In contrast, Kv4.3 and DPP10 may contribute to A-type current in non-peptidergic, C-fiber somatic afferent neurons.

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Keywords

Dorsal root ganglion; Voltage-gated potassium channel; Kv4.1; KChIP; DPP

Introduction

Voltage-gated K⁺ (Kv) currents in sensory neurons are divided into two major categories; sustained delayed rectifier (K_{DR}) and transient A-type K⁺ (K_A) currents (Kostyuk et al. 1981; Hall et al. 1994; Gold et al. 1996; Yoshimura et al. 1996). K_A current is activated at subthreshold of action potential and rapidly inactivates. Thus, this current is important to determine the initiation and interval of action potentials. K_A current in sensory neurons may be carried by a number of Kv pore-forming subunits including Kv1.4 and any of Kv4 subunits (Kv4.1, Kv4.2, and Kv4.3). It has been shown that Kv1.4 is localized in small-sized C-fiber DRG neurons (Rasband et al. 2001). Furthermore, K_A current in small-sized C-fiber neurons exhibits slower inactivation and sensitivity to α -dendrotoxin, a blocker of Kv1-family channels. In addition, reduced K_A current and Kv1.4 proteins are associated with hyperexcitability of DRG neurons in animal models of bladder pain (Hayashi et al. 2009). Therefore, Kv1.4 significantly contributes to the formation of A-type channels in a subset of C-fiber neurons. In contrast to Kv1.4 subunits, relatively less is known about cellular distributions of Kv4 channel subunits in DRGs. Previous studies showed that Kv4.3 protein is predominantly expressed in non-peptidergic, small-sized DRG neurons (Chien et al. 2007). PCR analysis also detected Kv4.1 mRNA in DRG tissue and a large number of isolated, small to medium-sized DRG neurons (Phuket and Covarrubias 2009). These findings support differential expression of the two Kv4 pore-forming subunits in distinct DRG neurons. Yet, the cell-size distribution of Kv4.1 in the entire DRG neuronal population remains unclear.

Kv4 pore-forming proteins are known to form complexes with two distinct types of auxiliary subunits that markedly alter channel expression and gating. The first type of Kv4 auxiliary subunits are small cytosolic Ca²⁺-binding proteins, namely Kv channel interacting proteins (KChIPs) (An et al., 2000), whereas the other type contains one transmembrane domain with a large extracellular portion similar to dipeptidyl peptidase (DPP6/10) (Jerng et al. 2004; Nadal et al. 2003; Ren et al. 2005). Diverse KChIPs are generated by the presence of four genes (An et al. 2000; Morohashi et al. 2002) and alternative splicing of transcripts (Rosati et al. 2001; Takimoto et al. 2002; Holmqvist et al. 2002; Patel et al. 2002; Boland et al. 2003). However, less is known about the distribution of KChIPs and DPPs in DRG neurons.

We wished to determine cellular distributions and subunit compositions of Kv4 channel complexes in distinct DRG neurons. We utilized PCR analysis, in-situ hybridization and immunohistochemistry to examine the expression and cellular distributions of Kv4 pore-forming and auxiliary subunits in rat DRG neurons.

Materials and Methods

Experiments were performed using female Sprague Dawley rats (220-250g). Care and handling of animals were in accordance with institutional guidelines and were approved by the Animal Care and Use Committees of the Nara Medical University and University of Pittsburgh Institutional Animal Care and Use Committees.

PCR analysis

Total RNAs were prepared from L6-S1 DRGs and total brain using a column-based isolation method (Qiagen, Valencia CA). Synthesis of cDNA was performed as described previously

(Takimoto et al., 2002). These primers were designed to detect splicing variants in different sizes (Table 1). PCR was done under the following conditions: denature at 94 °C for 5 seconds, annealing at 64 °C for 5 seconds and extension at 72 °C for 60 seconds for 28 cycles (22 cycles for GAPDH), and final extension at 72 °C for 4 minutes. PCR products were separated on a 5% polyacrylamide gel and stained with ethidium bromide for visualization. Control PCRs using cDNA made without reverse transcriptase generated no visible products.

Immunohistochemistry

One week after the Fluorogold (FG) injection into the bladder, the rats were perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, and L4 and L5 DRGs were then removed. Tissues were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight, and then cryoprotected in 10, 20, and 30% series of sucrose in 0.01 M phosphate buffered saline. Tissue was cut in 5 or 10 µm thick sections for subsequent histological examination. Immunohistochemical analyses were performed on tissue sections obtained from different DRGs.

The general expression pattern of Kv4 subunits were examined in L4 or L5 DRG sections. After quenching of endogenous peroxidase activity by using 3% hydrodioxyde, the tissue sections were incubated with 5% of bovine serum albumin in 0.01 M phosphate-buffered saline at room temperature for 30 minutes. The sections were then probed with antibody against Kv pore-forming subunits (anti-Kv4.2 antibody at the dilution 1: 100 or anti-Kv 4.3 antibody at 1: 200; NeuroMab Facility, Davis, California) in 5% of bovine serum albumin and 0.3% Triton X-100 in 0.01 M phosphate-buffered saline overnight at 4 °C. Kv immunoreactive proteins were detected with 5 mg L⁻¹ (1:200) biotin-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) at room temperature for 1 hour and then reacted with avidin-biotin peroxidase complex (VECTSTAIN Elite ABC Kit, Vector Laboratories, Inc., Burlingame, California) at room temperature for 30 minutes. The probed sections were washed with 0.02 M Tris-HCl pH 7.4 and incubated with diaminobenzidine (DAB Substrate kit, Vector Laboratories, Inc.). For immunofluorescence staining, anti-Kv4.3 (1: 200) or anti-KChIP3 (1: 200) antibody (NeuroMab Facility) in 5% of bovine serum albumin and 0.3% Triton X-100 in 0.01 M phosphate-buffered saline were applied overnight at 4 °C. The sections were incubated with 5 mg L⁻¹ (1:200) biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 2 hours and then reacted with 2.7 mgL⁻¹ Alexa Fluor 488-streptavidin conjugate (1:750) (Molecular probes Inc., Eugene, Oregon) at room temperature for 2 hours.

In situ hybridization

A portion of rat Kv4.1, Kv4.3, DPP6 and DPP10 cDNAs was obtained by RT-PCR and cloned into a topoisomerase-based vector (pCR2.1-TOPO or pCRII-TOPO, Invitrogen) for RNA probe synthesis. Primers used for the cloning were as follows: Kv4.1 5'-cacagacgagctaacttcag-3' and 5'-tcacaggaagagatcttgac-3' (GenBank ID: 116695); Kv4.3 5'-tgggttatcctatcttgga-3' and 5'-ttacaagacagaccttgac-3' (GenBank ID: 65195); DPP6, 5'-agcaatgacaacatccagtc-3' and 5'-agtaccatccaccagagc-3' (GenBank ID: 29272); DPP10 5'-gagcaaattacggtgcgcgact-3' and 5'-cttcattattattagtagaagagc-3' (GenBank Accession # AY_557199). Digoxigenin-labeled RNA probes were synthesized using linearized plasmids with T7 and SP6 RNA polymerases.

In situ hybridization was performed according to the procedure described previously (Tatsumi et al. 2005) and the signal was detected by dig-NBT/BCIP system. Briefly, after rehydration with 0.1M phosphate buffer, the sections of L4 DRG were treated with 0.2 M

HCl. The sections were then treated with $10 \mu\text{g mL}^{-1}$ proteinase K in 50 mM Tris-HCl and 5mM EDTA and then fixed with 4% formaldehyde in 0.1 M phosphate buffer. The sections were acetylated by 0.25% acetic anhydride in 0.1M triethanolamine, dehydrated by ethanol series (70, 95 and 100%), defatted in chloroform, rinsed with ethanol, and dried. Denatured labeled RNA probes were applied and hybridized at 50 °C. Remaining probes were eliminated with RNase A, followed by washing with 50% formamide in sodium chloride sodium citrate. Digoxigenin-labeled probes were processed by anti-digoxigenin antibody-conjugated alkaline phosphatase, and visualized using nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt.

Histological analysis

Sections stained by *in situ* hybridization technique and immunohistochemistry with DAB were viewed under an Olympus BX51 Microscope (OLYMPUS Corp., Tokyo, Japan) in bright field. Fluorescent images were captured on an Olympus FluoView 1000 confocal microscope (OLYMPUS Corp., Tokyo, Japan). Randomly selected two sections from each DRG at more than 50 μm intervals were used for counting of positively stained cells to avoid double counting of cells. Cross-sectional areas of all neuronal profiles, in which nuclei were identified, were measured by using Scion Image (Scion Corp., Frederick, Maryland). Neuronal profiles were then divided into small-, medium- and large-sized neuronal populations based on the area size (area<600 μm^2 , 600 μm^2 <area<1200 μm^2 and area>1200 μm^2 , respectively). The staining intensity was rated on a four point scale, from completely negative (grade 0) to intense staining (grade 3), and the neurons that exhibited grades 2 or 3 were regarded as positively stained cells. The number of positively stained cells as well as the total DRG neurons was counted, and the percent ratio of positively stained cells against the total DRG cells was calculated.

Results

Cellular distributions of Kv4 pore-forming subunits in DRGs

Previous PCR analysis suggested significant expression of Kv4.1 and Kv4.3 mRNAs in DRG neurons (Phuket and Covarrubias, 2009). We also observed abundant Kv4.1 and long-isoform of Kv4.3 transcripts, but not Kv4.2 mRNA, in L6-S1 DRGs (unpublished observation). Thus, we first examined the cellular distributions of the two pore-forming subunits, Kv4.1 and Kv4.3. Since commercial anti-Kv4.1 antibodies appeared less suitable for immunohistochemistry, we used *in situ* hybridization to test for differential distributions of Kv4.1 and Kv4.3 in DRG neurons (Fig. 1). Antisense Kv4.3 probe preferentially stained small-sized neuronal cell bodies (Fig. 1B). Kv4.3 transcript-positive cells represented approximately 32.8% of DRG neurons (total 4 sections), equivalent to that of the corresponding channel proteins (33.8%). Most Kv4.3 mRNA-positive cells were less than 900 μm^2 in cell area size. Consistent with mRNA distribution, anti-Kv4.3 antibody stained small-sized neuronal cell bodies (data not shown). Kv4.3 protein-positive cells represented approximately 33.8% of DRG neurons.

In contrast, antisense Kv4.1 probe detected DRG neurons in various sizes (Fig. 2A). Kv4.1 mRNA-positive cells represented 59.5 % of DRG neurons (total 4 sections) and were distributed in all sizes. Thus, the two Kv4 pore-forming subunits are differentially distributed in DRG neurons.

Cellular distributions of Kv4 channel auxiliary subunits

Kv4 pore-forming subunits may be associated with the two distinct types of auxiliary subunits that significantly alter channel expression and gating. We first tested expression of KCHIP 1-4 mRNAs by RT-PCR analysis (Fig. 2A). All four gene transcripts were abundant

in the brain, whereas only KChIP3 mRNA was significant in L6-S1 DRGs. Immunostaining with anti-KChIP3 antibody showed that KChIP3 proteins were prominent in a subset of medium to large-sized neuronal cell bodies (Fig. 2B and C). Staining with anti-Kv4.3 antibody of adjunct sections suggested that Kv4.3 and KChIP3 are not colocalized in the same cells.

We next examined cellular distribution of the other type of Kv4 channel auxiliary subunits, DPP6 and DPP10. Our previous study demonstrated abundant mRNA expression of these two auxiliary subunits in DRGs (Takimoto et al. 2006). Since several available antibodies against these proteins failed to provide reliable staining, we performed *in situ* hybridization analyses (Fig. 3). DPP6 mRNA was widely distributed in DRG neuronal cell bodies in all sizes (Fig. 3A), whereas DPP 10 transcript was expressed mostly in small to medium-sized neurons ranging 400-1600 μm^2 of cell area size (Fig. 3B). The proportions of DPP6 and DPP 10 mRNA-positive cells among DRG neurons per section were 72.8% and 27.2%, respectively (the mean of n=2 sections). Thus, the two auxiliary subunits differently contribute to the production of Kv4 channel complexes in DRG neurons with different sizes.

Discussion

DRGs contain cell bodies for heterogeneous populations of primary afferent neurons. These neurons may be categorized by cell body sizes and innervating tissues. A α / β -fiber neurons with large-sized cell bodies generally carry mechanical information, whereas small-sized cell bodies correspond to C and A δ -fiber neurons that are responsible for pain sensation. The latter small-sized neurons are also implicated in the development of chronic pain. In this study, we determined the cellular distribution of Kv4 pore-forming subunits and their associating auxiliary subunits in DRG neurons. We found that Kv4.1 mRNA is widely expressed in DRG neurons with various cell body sizes. Similarly, mRNA for the auxiliary subunit DPP6 is ubiquitous in neurons with various cell body sizes, whereas DPP 10 transcript is more concentrated in small-sized neurons. In addition, KChIP3 mRNA seems more abundant in medium to large-sized neurons. These new findings suggest that Kv4.1 channel complexes containing DPP6 and KChIP3 contribute to K_A currents in medium to large-sized A-fiber neurons, whereas Kv4.3 channel complexes containing DPP 10 may be responsible for K_A currents in small-sized C-fiber somatic neurons.

DRG neurons are known to contain two types of K_A currents with distinct kinetics (fast vs. slow-inactivating) and toxin sensitivities. Fast-inactivating K_A current is sensitive to heteropodatoxins and phrixotoxins that influence the gating of Kv4 channels, but not Kv1 or Kv2 channels (Sanguinetti et al. 1997; Diochot et al. 1999; Escoubas et al. 2002). Thus, it is assumed that Kv4 channel complexes are responsible for the fast K_A current. While fast-inactivating K_A current is prominent in medium to large-cell sized A-fiber neurons (Gold et al. 1996; Yoshimura and de Groat 1996), it may also be present in a subset of small-sized C-fiber neurons. Our immunostaining and *in situ* hybridization clearly showed the presence of Kv4.3 pore-forming subunit in IB4-positive, small-sized somatic DRG neurons and DPP10 auxiliary subunit in small-sized DRG neurons, respectively. We have recently observed that phrixotoxin-sensitive K_A current is prominent in somatic sensory neurons, but not bladder afferent cells (unpublished observation). Thus, Kv4.3 pore-forming and DPP10 ancillary subunits may contribute to the formation of fast K_A channels in somatic C-fiber neurons. In addition, Kv4.1 mRNA has been detected in dissociated, small to medium-cell sized DRG neurons (Phuket and Covarrubias 2009). Our *in situ* hybridization study further demonstrated that Kv4.1 mRNA is expressed not only in small to medium-sized DRG neurons, but in DRG neurons with various cell sizes. Therefore, it is likely that this Kv4.1 pore-forming subunit may also participates in forming fast K_A channels in small-sized DRG neurons.

Kv4 channels may simultaneously contain the two distinct auxiliary subunits, KChIPs and DPP6/10. In the brain, immunoprecipitation studies indicated ternary channel complexes containing the two types of auxiliary subunits (Jerng et al. 2005; Amarillo et al. 2008). However, our RT-PCR analysis and *in situ* hybridization suggest that some Kv4 channel complexes may not contain KChIPs. RT-PCR analysis detected a high level of KChIP3 without apparent expression of other three KChIPs in DRGs, whereas all four auxiliary subunit mRNAs were abundant in the brain. Moreover, immunohistochemistry indicated that KChIP3 protein is present in medium to large-sized neurons, but not in small-sized cells. A simple explanation for these observations is that Kv4 channel complexes in small-sized DRG neurons consist of Kv4.1/Kv4.3 and DPP6/10, but not any KChIPs, whereas Kv4 channel complexes in medium to large-sized DRG neurons consist of Kv4.1, DPP6 and KChIP3. Heterologous expression studies suggest that KChIPs and DPP6/10 somewhat play redundant roles in raising expression of the associated pore-forming subunits and inducing faster recovery from inactivation. Therefore, it is possible that a subset of small-sized C-fiber neurons contain Kv4 channel complexes without any KChIPs.

Sensory neuron-type selective expression of different channel subunits may provide the basis for the development of new therapeutic strategy or drugs for chronic pain and other disorders. We have previously showed that reduced expression of Kv1.4 subunits is associated with hyperexcitability of DRG neurons in an animal model of bladder inflammation (Hayashi et al. 2009). In contrast to visceral pain, less attention is focused on molecular correlates for Kv channel plasticity in primary afferents that transmit somatic pain, such as arthritis and chronic back pain. Further studies on alterations in the expression of Kv4 pore-forming and auxiliary subunits and functional properties of Kv4-mediated K_A currents could identify the molecular correlates that contribute to somatic pain conditions.

Conclusion

Kv4 channel complexes in small-sized, somatic DRG neurons consist of Kv4.1/Kv4.3 and DPP6/10, but not any KChIPs, whereas Kv4 channel complexes in medium to large-sized DRG neurons consist of Kv4.1, DPP6 and KChIP3.

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Fig. 1. Cellular distributions of Kv4.1 and Kv4.3 mRNA in rat DRGs

A. Rat DRG sections stained with Kv4.1 -sense RNA probe (a) or Kv4.1 -antisense RNA probe (b), are shown. Cell-size distributions are shown with filled and open columns indicating Kv4.1 mRNA-positive and total DRG neuronal cell bodies, respectively (c). B. In situ hybridization picture and cell-size distributions for Kv4.3 are shown. Scale bar, 50 μm . Bin width: 100 μm^2 .

Fig. 2. Expression of KChIPs in rat DRGs

A. RT-PCR data with primers for KChIP1-4 in the brain and L6-S1 DRG are shown. Different sizes of bands represent splicing variants: KChIP1a and KChIP1b (Boland, et al., 2003); KChIP2a, KChIP2b and KChIP2c (Takimoto, et al., 2002); KChIP4 (Takimoto, et al., 2002). Note that DRGs contain only KChIP3. B. Adjacent L5 DRG sections at a 10 μm interval stained with anti-Kv4.3 antibody (a) or anti-KChIP3 antibody (b) are shown. Scale bar, 50 μm . Arrow heads (a) and arrows (b) indicate the same cells that are negative for Kv4.3 and positive for KChIP3, respectively. Cell-size distributions are shown with filled and open columns indicating KChIP3 mRNA-positive and total DRG neuronal cell bodies, respectively (c). Bin width: 100 μm^2 .

Fig. 3. Cellular distributions of DPP6 or DPP10 mRNA in rat DRGs

A. Rat DRG sections stained with DPP6-sense (a) or antisense RNA probe (b), are shown. Cell-size distributions are shown with filled and open columns indicating DPP6 mRNA-positive and total DRG neuronal cell bodies, respectively (c). B. In situ hybridization picture and cell-size distributions for DPP10 are shown. Scale bar, 50 μm . Bin width: 100 μm^2 .

Table 1
Primers used in the RT-PCR study

Gene	GenBank Accession #	Sequence 5'-3'	Position	Product sizes
KChIP1	AY082657	atggggccgctcatgggcac accacaccactggggcactcg	1 -20 239-219	KChIP1a 239 KChIP1b 206
KChIP2	XM_342059	gtcctatgaccagcttacgg cctcgttgacaatcccactgg	275-295 598-578	KChIP2a 324 KChIP2b 270 KChIP2c 174
KChIP3	NM_032462	aggctcagacagcagtgaca gggggaagaactgggaataa	197-216 393-374	197
KChIP4	AF345444	agatgaacgtgagaagggtgga gtgcatatgtgtggagtctc	376-397 753-733	KChIP4a 378 KChIP4b 276
GAPDH	NM_017008	gccatcactgccactcag gtgagctcccgttcagc	608-625 739-756	149