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R-type Calcium Channel Isoform in Rat Dorsal Root Ganglion Neurons

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R-type $Ca_v2.3$ high voltage-activated Ca^{2+} channels in peripheral sensory neurons contribute to pain transmission. Recently we have demonstrated that, among the six $Ca_v2.3$ isoforms ($Ca_v2.3a \sim Ca_v2.3e$), the $Ca_v2.3e$ isoform is primarily expressed in trigeminal ganglion (TG) nociceptive neurons. In the present study, we further investigated expression patterns of $Ca_v2.3$ isoforms in the dorsal root ganglion (DRG) neurons. As in TG neurons, whole tissue RT-PCR analyses revealed the presence of two isoforms, $Ca_v2.3a$ and $Ca_v2.3e$, in DRG neurons. Single-cell RT-PCR detected the expression of $Ca_v2.3e$ mRNA in 20% (n=14/70) of DRG neurons, relative to $Ca_v2.3a$ expression in 2.8% (n=2/70) of DRG neurons. $Ca_v2.3e$ mRNA was mainly detected in small-sized neurons (n=12/14), but in only a few medium-sized neurons (n=2/14) and not in large-sized neurons, indicating the prominence of $Ca_v2.3e$ in nociceptive DRG neurons. Moreover, $Ca_v2.3e$ was preferentially expressed in tyrosine-kinase A (trkA)-positive, isolectin B4 (IB4)-negative and transient receptor potential vanilloid 1 (TRPV1)-positive neurons. These results suggest that $Ca_v2.3e$ may be the main R-type Ca^{2+} channel isoform in nociceptive DRG neurons and thereby a potential target for pain treatment, not only in the trigeminal system but also in the spinal system.

Key Words: R-type calcium channels, $Ca_v2.3$, Voltage-activated calcium channels, DRG neurons

INTRODUCTION

The high voltage-activated Ca^{2+} channels (HVACCs) play a key role in cellular process under physical, chemical and inflammatory damage in peripheral tissues [1]; they are classified into L-, N-, P/Q- and R-type Ca^{2+} channels on the basis of pharmacological and electrophysiological properties in sensory neurons such as dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons [2,3]. HVACCs are formed by one of a number of pore-forming α_1 subunits, $\alpha_{1A\sim F}$ and α_{1S}, in addition to auxiliary subunits. Molecular characterizations have determined that α_{1C}, α_{1D}, α_{1F}, and α_{1S} subunits encode L-type ($Ca_v1.1$, 1.2, 1.3 and 1.4) channels [4]; α_{1A} encodes P/Q-type ($Ca_v2.1$) channels [5]; α_{1B} encodes N-type ($Ca_v2.2$) channels [6]; α_{1E} encodes R-type ($Ca_v2.3$) channels [7].

Six isoforms of the R-type $Ca_v2.3$ Ca^{2+} channel ($Ca_v2.3a \sim Ca_v2.3f$) have been reported in various mammalian species [8-12]. R-type Ca^{2+} channels are also present in DRG neurons [2], and have been suggested to play a critical role in the pain transmission and neuropathic pain [13,14]. Immunohistochemical and *in situ* hybridization analysis also showed differential expression of R-type Ca^{2+} channels in DRG neurons [15,16]. In the previous study, we demonstrated that $Ca_v2.3e$ is the major $Ca_v2.3$ isoform in the nociceptive TG neurons, the counterpart of DRG neurons in the orofacial area [17]. However, to date, expression patterns of R-type $Ca_v2.3$ Ca^{2+} channel in DRG neurons have not been characterized.

In the present study, we determined the $Ca_v2.3$ isoforms uniquely expressed in rat nociceptive DRG neurons by using whole-tissue RT-PCR and single-cell RT-PCR. Our results show that $Ca_v2.3$ isoform in nociceptive DRG neurons is comparable to that of TG neurons; $Ca_v2.3e$ is the major R-type $Ca_v2.3$ isoform in nociceptive DRG neurons and is preferentially expressed in IB4-negative, trkA-positive and TRPV1-positive neurons.

METHODS

Preparation of DRG neurons

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) in School of Dentistry, Seoul National University. DRG neurons from 2- to 5-day old neonatal rats were prepared as previously described [18]. Briefly, DRGs were rapidly removed from spinal segments and digested sequentially in collagenase (Sigma, St. Louis, MO), dispase (Boe-

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ABBREVIATIONS: DRG, dorsal root ganglion; IB4, isolectin B4; HVACCs, high voltage-activated calcium channels; RT-PCR, reverse transcription-polymerase chain reaction; TG, trigeminal ganglion; trkA, tyrosine kinase A; TRPV1, transient receptor potential vanilloid 1.

hringer Mannheim, Indianapolis, IN), and trypsin (Life Technologies) in HBSS for 10 min at 37°C. The cells were washed in DMEM, triturated with a flame-polished Pasteur pipette to separate cells and remove processes. Subsequently, cells were centrifuged and resuspended, and then placed on a polyornithine-coated glass coverslips (25 mm in diameter). Cells were maintained in an incubator at 37°C equilibrated with 5% CO_2. All experiments were performed with cells cultured for 4~6 h.

Whole tissue Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from 2- to 5-day-old rats DRG by using the Trizol reagent (Life Technologies). Following digestion with DNase I, 3 μg of total RNA was used for cDNA synthesis using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, California). After reverse transcription of total RNA, 1 ng of cDNA was then used as a template for amplification in a reaction mixture. As shown in our previous study [17], primers for PCR were specifically designed to differentiate the presence or the absence of three inserts (inserts I, II and III) on the $Ca_v2.3$ transcripts. After a denaturation step of 5 min at 94°C, the amplification was carried out at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s for 35 cycles. The PCR reaction was completed by maintaining temperatures at 72°C for 10 min. As a positive control, cDNA from the same preparations was subjected to 35 cycles of PCR with primers for β-actin. All PCR products were resolved on 2% agarose gel.

Single-cell RT-PCR

The entire single cell was aspirated into a pipette under visual control via negative pressure. Pipettes used for entire neuron harvest had a tip diameter range of 12~30 μm, and was filled with RNase-free water. The tip of the pipette and its contents were broken into a reaction tube containing reverse transcription (RT) reagents. To avoid genomic DNA contamination, digestion with DNase was performed before RT. RT was carried out for 1 h at 50°C (Invitrogen) and the cDNA product was used in separate PCR. The forward and reverse primers were chosen from parts specific to the gene to be detected in order to avoid amplification of closely homologous genes. The first round of PCR was preformed in 50 μl of PCR buffer containing 0.2 mM dNTPs, 0.2 μM "outer" primers, 5 μl of RT product, and 0.2 μl of platinum Taq DNA polymerase (Invitrogen). The protocol included 5 min of initial denaturation step at 95°C, followed by 40 cycles of 40 s of denaturation at 95°C, 40 s of annealing at 55°C, 40 s of elongation at 72°C, and was completed with 7 min of final elongation. For the second round of amplification, the reaction buffer (20 μl) contained 0.2 mM dNTPs, 0.2 μM "*inner*" primers, 5 μl of the products from the first round PCR, and 0.1 μl of platinum Taq DNA polymerase. The reaction procedure was the same as the first round. "*Insert*" primers, designed to detect the presence of insert fragments, were also used in second round amplifications. For the positive controls, β-actin primers were used in parallel in each PCR reaction. Negative control was obtained from pipettes that did not harvest any cell contents but were submerged in bath solution. The PCR products were displayed on ethidium bromide-stained 2% agarose gel. Gels were photographed using a digital camera (Bio-print 2000 x-press zoom, Vilber Lourmat, France).

Classification of sensory neurons

DRG neurons are classified into three groups, small-sized (<16 μm), medium-sized (16~20 μm) and large-sized (20 ~30 μm) neurons [19]. *Griffonia simplicifolia* isolectin B4 (IB4) was also utilized to classify DRG neurons into either IB4-positive or IB4-negative neurons [20]. Before single cell collection, DRG cells were incubated with 10 μg/ml IB4-FITC (Sigma) in a balanced salt solution [(in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose] for 10 min and then rinsed.

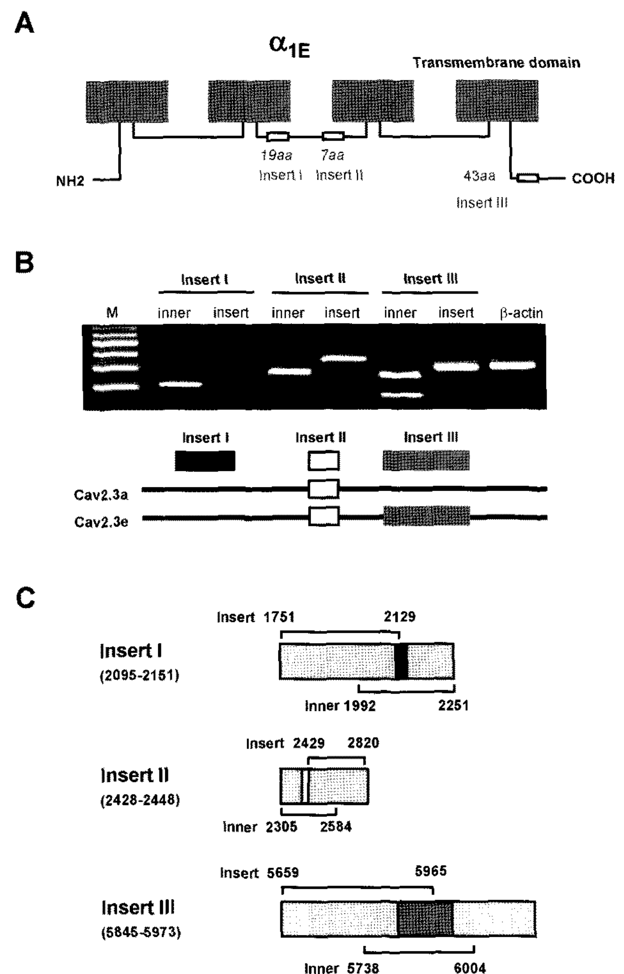

Fig. 1. (A) Schematic diagram of α_{1E} ($Ca_v2.3$) subunit. The structural variations cover two segments of 19 (insert I) and 7 amino acids (insert II) in the loop between domain II and III, and a third segment of 43 amino acids (insert III) in the proximal carboxy terminus. (B) Two $Ca_v2.3$ isoforms were found in DRG. Insert I, II and III was analyzed by whole tissue RT-PCR. $Ca_v2.3$ isoforms amplified from DRG neurons have insert II, but not insert I, and either lack or contain insert III depending on the isoform. $Ca_v2.3a$ contains insert II, but not insert I and insert III, while $Ca_v2.3e$ has insert II and insert III, but not insert I. (C) Illustrated are the locations of the primers designed to detect insert I, insert II and insert III for RT-PCR analysis in relation to the $Ca_v2.3$ subunit.

RESULTS

Presence of $Ca_v2.3$ isoforms $Ca_v2.3a$ and $Ca_v2.3e$ in DRG neurons

In order to determine $Ca_v2.3$ isoform in DRG neurons, we utilized the same strategy, as previously done in TG neurons [17], which examined the presence or the absence of three major inserts: insert I, insert II in the II-III loop, and insert III in the carboxy terminus (Fig. 1A). Using both "*inner*" primers and "*insert*" primers, the PCR products were differentiated by size and additionally by the presence of the insert region (Fig. 1C). For the II-III loop, the PCR amplification only yielded $Ca_v2.3$ isoforms containing insert II but lacking insert I. However, PCR amplification of the carboxy terminus we could detect both shorter cDNA fragments that lack insert III and longer cDNA fragments that contain insert III. These two isoforms were $Ca_v2.3a$ and $Ca_v2.3e$ splice variants, respectively (Fig. 1B).

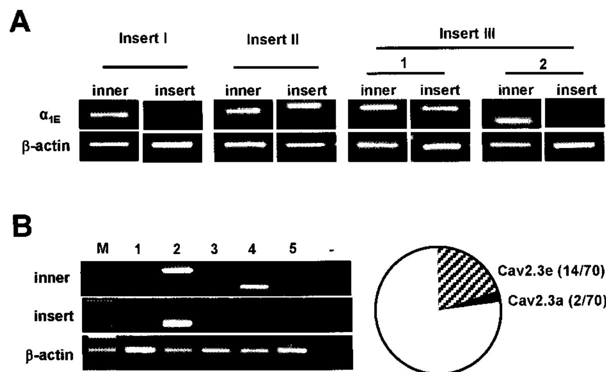

Fig. 2. Expression of $Ca_v2.3a$ and $Ca_v2.3e$ isoforms from individual DRG neurons. (A) Single-cell RT-PCR products amplified with nested primer from DRG neurons. PCR products, with or without insert III (larger, 1 and smaller, 2), were produced in each DRG neuron. (B) Representative gels showing single-cell RT-PCR products amplified using insert III-specific primers. The numbers (1-5) indicate five different neurons examined from single-cell RT-PCR reaction. β-actin was used in each reaction as a positive control. Circle diagram shows the distribution of $Ca_v2.3a$ and $Ca_v2.3e$ in DRG neurons (total 70 neurons).

The prominance of $Ca_v2.3e$ in DRG neurons

Given that only two isoforms of $Ca_v2.3$-$Ca_v2.3a$ and $Ca_v2.3e$ splice variants were present in DRG, we then determined the expression patterns of these two isoforms at the single cell level. By using single-cell RT-PCR, we detected $Ca_v2.3a$ and $Ca_v2.3e$ mRNA in subpopulations of DRG neurons, which was in good agreement with whole-tissue RT-PCR (Fig. 2A). Since $Ca_v2.3a$ and $Ca_v2.3e$ differ only in the presence or absence of insert III, we analyzed insert III to discriminate their expression in individual cells. $Ca_v2.3e$ and $Ca_v2.3a$ mRNAs were detected in 20.0% (n=14) and 2.8% (n=2) of neurons, respectively (n=70; Fig. 2B). Likewise in TG neurons, we found that $Ca_v2.3e$ was the prominant isoform of $Ca_v2.3$ in DRG neurons but did not observe any DRG neurons that co-expressed both $Ca_v2.3e$ and $Ca_v2.3a$ in the same cells.

Preferential Expression of $Ca_v2.3e$ isoform in trkA-positive, IB4-negative and TRPV1-positive small-sized DRG neurons

Neonatal DRG neurons can be classified into small-, medium- and large-sized neurons, with the small-sized neurons functioning as nociceptors [21]. $Ca_v2.3e$ mRNA was detected in 30.0% (n=12/40) of small-sized neurons, 13.3% (n=2/15) of medium-sized neurons, and 0% (n=0/15) of large-sized neurons (Fig. 3). These results indicate that $Ca_v2.3e$ is prominently expressed in nociceptive DRG neurons as $Ca_v2.3e$ mRNA was mainly detected in small-sized neurons (n=12/14), but in only a few medium-sized neurons (n=2/14) and not in large-sized neurons.

Small-diameter sensory neurons can be divided into two types based on their neurochemical properties [22]. One group contains neuropeptides such as calcitonin-gene related neuropeptide (CGRP) and substance P, and express the high-affinity nerve growth factor (NGF) receptor trkA; the other group lacking neuropeptides instead possess fluoride-resistant acid phosphatase (FRAP) activity, bind the plant lectin isolectin B4 (IB4) and are positive for the ATP-gated ionotropic receptor $P2X_3$ [23-25]. We used an IB4-FITC conjugate to distinguish IB4-positive small-sized DRG neurons (n=40). IB4-positive neurons (80% of which expressed $P2X_3$ mRNA) did not contain $Ca_v2.3e$ mRNA (n=10). Of the small diameter IB4-negative neurons, 80% expressed trkA mRNA; $Ca_v2.3e$ (n=12/24) was only detected in trkA-positive neurons (Fig. 4A).

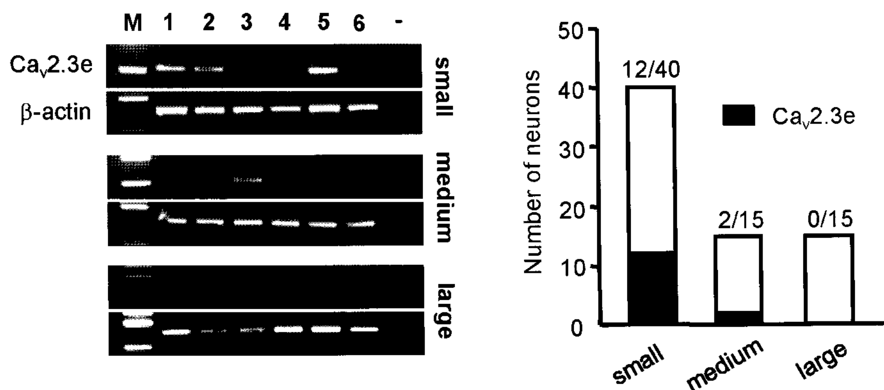

Fig. 3. Expression pattern of $Ca_v2.3e$ was analyzed in three groups: small-sized (<16 μm), medium-sized (16~20 μm) and large-sized (20~30 μm) DRG neurons. Representative gels showing single-cell RT-PCR products obtained from six different neurons. White bars indicate the number of neurons in each group; black bars indicate the number of neurons with expression of $Ca_v2.3e$.

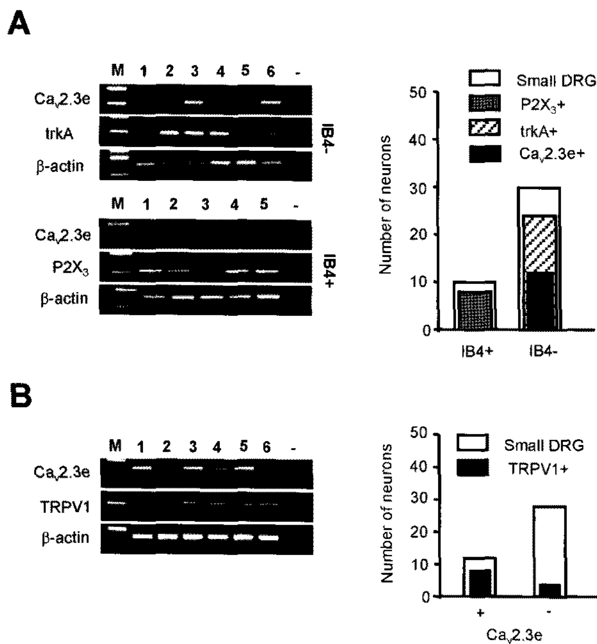

Fig. 4. (A) Small-sized DRG neurons were divided into IB4-negative and IB4-positive neurons; expression of $Ca_v2.3e$ was determined in each group with or without trkA and $P2X_3$ expression. The black bar in the graph shows that $Ca_v2.3e$ is prominent in trkA+/IB4-/TRPV1+ neurons. (B) Expression of $Ca_v2.3e$ in TRPV1-expressing nociceptive neurons. Representative gels showing RT-PCR products amplified with $Ca_v2.3e$, TRPV1, and β-actin-specific primers from six different neurons. The majority of small-sized $Ca_v2.3e$-expressing DRG neurons are also TRPV1-positive, though TRPV1 expression is not exclusive to DRG expressing the $Ca_v2.3e$ subunit (graph; right).

We also examined whether $Ca_v2.3e$ mRNAs are expressed together with TRPV1 in small neurons. 66.7% (n=8/12) of neurons expressing $Ca_v2.3e$ mRNA were TRPV1-positive, while 14.3% of neurons lacking $Ca_v2.3e$ mRNA were TRPV1-negative (Fig. 4B).

DISCUSSION

Our recent study revealed that $Ca_v2.3e$ expression is highly restricted to small (<16 μm) IB4-negative, trkA-positive, and TRPV1 TG neurons [17]. In the present study, we determined expression patterns of $Ca_v2.3$ isoform in DRG neurons. We found that $Ca_v2.3a$ and $Ca_v2.3e$ isoforms were present in DRG neurons. Among these two isoforms, $Ca_v2.3e$ was the major isoform preferentially expressed in small-sized TRPV1-positive nociceptive neurons and was restricted to IB4-negative and trkA-positive neurons. These results suggest that the expression patterns of $Ca_v2.3$ isoforms in nociceptive DRG neurons are similar to those of TG neurons; the strong presence of $Ca_v2.3e$ mRNA may correlate with the expression of the R-type Ca^{2+} channel, which might play a critical role in pain transmission in DRG neurons [26].

Among six isoforms ($Ca_v2.3a$, $Ca_v2.3b$, $Ca_v2.3c$, $Ca_v2.3d$, $Ca_v2.3e$, $Ca_v2.3f$), $Ca_v2.3e$ is the major endocrine $Ca_v2.3$ isoform that was identified in rat and human kidney, insulinoma cell line INS-1 cells and islets of Langerhans [10]. We have shown that $Ca_v2.3e$ is uniquely expressed in trkA-positive, IB4-negative DRG neurons that contain neuropeptide transmitters such as substance P and CGRP. Based on these results, $Ca_v2.3e$ might be an important molecular mediator in neuropeptide release from nociceptive nerve terminals, in which case $Ca_v2.3e$ may be the major $Ca_v2.3$ isoform responsible for the nociception mediated by $Ca_v2.3$ in the spinal system.

In accordance with our previous work in TG neurons [17], $Ca_v2.3e$-expressing DRG neurons also exhibited several properties of nociceptors, such as being of small-diameter, IB4-negative and positive for TRPV1 and trkA. It has been reported that α_{1E} subunits were heterogeneously localized in the cell bodies of the DRG neurons in immunohistochemistry [15] and *in situ* hybridization [16]. $Ca_v2.3e$ expression at a higher level in the small-sized DRG neurons suggests that $Ca_v2.3e$ might have distinctive role in transduction of pain in nociceptive neurons. Also, restricted distribution of $Ca_v2.3e$ to trkA-positive/IB4-negative nociceptors, but not to P2X3-positive/IB4-positive neuron, suggests that these two populations may transmit pain information to the spinal cord in different manners that are possibly mediated by the participation of different Ca^{2+} channel isoforms. However, it remains to be determined the exact functional role of preferential $Ca_v2.3e$ expression in trkA-positive/IB4-negative and TRPV1-positive neurons.

R-type Ca^{2+} channels are associated with nociceptive processing in various pain conditions. Mathews et al reported that spinal SNX-482, a $Ca_v2.3$ Ca^{2+} channel blocker, inhibited noxious C-fiber and Aδ-fiber-mediated dorsal horn neuronal responses in conditions of neuropathy but not in sham operated rats and that non-noxious Aδ-mediated responses were not affected by SNX-482 [13]. In another study, mice lacking the α_{1E} Ca^{2+} channel subunit (required for functional expression of $Ca_v2.3$ channels) exhibited normal pain behavior against acute mechanical, thermal, and chemical stimuli; however, they showed reduced responses to somatic inflammatory pain [15]. Also, R-type Ca^{2+} channels are located at primary synapses [27] and contributes to neurotransmitter release [28]. These results suggest that R-type Ca^{2+} channels may play a dominant role in neuropathic pain rather than in normal physiological pain. Further work is required to elucidate role of $Ca_v2.3e$ in the pathological pain conditions.

In summary, our study provides the first evidence that two $Ca_v2.3$ isoforms are expressed in rat DRG neurons, a major isoform ($Ca_v2.3e$) and a minor isoform ($Ca_v2.3a$). The expression pattern of $Ca_v2.3$ isoforms suggests that $Ca_v2.3e$ isoform of R-type Ca^{2+} channels may be important in pain transmission and neuropathic pain both in DRG neurons and TG neurons. Therefore, $Ca_v2.3e$ isoform in nociceptive neurons would be potential target for the pain treatment not only in the trigeminal system but also in the spinal system.

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