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Molecular Characterization of $Ca_V2.3$ in Rat Trigeminal Ganglion Neurons

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R-type ($Ca_V2.3$) calcium channel contributes to pain sensation in peripheral sensory neurons. Six isoforms of $Ca_V2.3$ that result from combinations of presence or deletion of three inserts (insert I and insert in the II-III loop, and insert III in N-terminal regions) have been demonstrated to be present in different mammalian tissues. However, the molecular basis of $Ca_V2.3$ in trigeminal ganglion (TG) neurons is not known. In the present study, we determined which isoforms of $Ca_V2.3$ are expressed in rat TG neurons using the RT-PCR analysis. Whole tissue RT-PCR analyses revealed that only two isoforms, $Ca_V2.3a$ and $Ca_V2.3e$, were present in TG neurons. From single-cell RT-PCR, we found that $Ca_V2.3e$ rather than $Ca_V2.3a$ was the major isoform expressed in TG neurons, and $Ca_V2.3e$ was preferentially detected in small-sized neurons that express nociceptive marker, transient receptor potential vanilloid 1 (TRPV1). Our results suggest that $Ca_V2.3e$ in trigeminal neurons may be a potential target for the pain treatment.

Keywords: $Ca_V2.3$, voltage-activated calcium channels, trigeminal ganglion, nociceptor

Introduction

Voltage-activated calcium channels (VACCs) have a variety of physiological functions regulating the firing pattern, synaptic modulation and neurotransmitter release in the nervous system (Catterall, 2000). It is generally accepted that all of these calcium channels are formed by one of a number of pore-forming α_1 subunits $\alpha_{1A\sim I}$ 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 channels; α_{1A} encodes P/Q-type channels; α_{1B} encodes N-type channels; α_{1E} encodes R-type channels; and α_{1G}, α_{1H}, and α_{1I} encode T-type Ca^{2+} channels (Catterall, 2000). R-type currents with diverse biophysical properties were described in different types of CNS neurons (Dunlap *et al.*, 1994; Williams *et al.*, 1994). However, the molecular nature of R-type currents in different types of native neurons is not fully understood yet (Wilson *et al.*, 2000; Lee *et al.*, 2002).

The association of a single α_1 subunit with multiple functionally distinct auxiliary subunits can generate channel complexes with more divergent properties (Catterall, 2000). In addition, the primary sequence itself can be the source of heterogeneity by undergoing extensive RNA processing, including alternate splicing (Dredge *et al.*, 2001; Graveley, 2001) and RNA editing (Keegan *et al.*, 2001). R-type calcium channels also contain alternatively spliced variants of $Ca_V2.3$ (α_{1E}). The structural variations are found in two segments, a 19 amino acid segment (insert I) and a 7 amino acid segment (insert II) in the loop between domain II and III, and in a third segment of 43 amino acids (insert III) in the proximal carboxy terminus. Genetic analysis of $Ca_V2.3$ revealed that insert I and insert III correspond to exon 19 and 45, respectively, and insert II is located within exon 20. To date, six isoforms ($Ca_V2.3a$, $Ca_V2.3b$, $Ca_V2.3c$, $Ca_V2.3d$, $Ca_V2.3e$, $Ca_V2.3f$), out of eight different isoforms which can be deduced from the $Ca_V2.3$ sequence, have been described in various mammalian species (Schneider *et al.*, 1994; Williams *et al.*, 1994; Vajna *et al.*, 1998; Schramm *et al.*, 1999; Pereverzev *et al.*, 2002). For example, $Ca_V2.3c$,

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$Ca_v2.3d$ and $Ca_v2.3f$ have been suggested as neuronal $Ca_v2.3$ isoforms, while $Ca_v2.3e$ was distributed in islets of Langerhans (Vajna *et al.*, 1998). In primary sensory neurons, $Ca_v2.3$ calcium channels have been suggested to play a critical role in the pain transmission (Saegusa *et al.*, 2000). Immunohistochemical and in situ hybridization analysis showed differential expression of α_{1E} calcium channels in DRG neurons (Murakami *et al.*, 2001; Yusaf *et al.*, 2001). However, expression patterns of α_{1E} calcium channel isoforms of $Ca_v2.3$ in primary sensory neurons have not been characterized.

The trigeminal ganglion (TG) neurons are involved in the transmission of orofacial sensory information (Sessle, 2002). Physical, chemical and inflammatory damage in peripheral tissues is a source of persistent nociceptive messages and gives rise to increased excitability of nociceptive neurons in the TG (Hokfelt *et al.*, 1994). The neuronal changes that underlie such circumstances involve processes in which calcium ions play a key role. Extracellular calcium enter the cell by way of several paths, the major one is the VACCs (Vanegas *et al.*, 2000). TG neurons display several types of high-voltage-activated calcium channel (HVACC) currents, which can be classified as L-, N-, P/Q- and R-type, on the basis of pharmacological and electrophysiological properties (Ikeda *et al.*, 2003).

In the present study, we determined unique $Ca_v2.3$ isoform expressed in the rat TG neuron by molecular biological approaches using whole tissue/single-cell RT-PCR. As a result, we found that $Ca_v2.3e$ is the major $Ca_v2.3$ isoform in TG neurons, and obtained positive evidence on the preferential expression of $Ca_v2.3e$ in small-sized (<16 μm) TRPV1-positive neurons.

Materials and Methods

Preparation of TG neurons

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in College of Dentistry, Seoul National University. TG neurons from 2- to 5-day old neonatal rats were prepared as previously described, respectively (Yang *et al.*, 2003). TG cells were washed several times in cold (4°C) HBSS (Life Technologies, Gaithersburg, MD), then incubated for 20 min at 37°C in HBSS containing trypsin. 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.

Whole tissue reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to analyze the expression of α_{1E} isoforms using total RNA derived from rat TG whole tissue. Total RNA has been isolated from 2- to 5-day-old rats TG 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) according to the manufacturer's instructions. After reverse transcription of total RNA, 1 ng of cDNA was then used as a template for amplification in a reaction mixture. 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 as described in Table 1, based on GenBank rat cDNA sequences. We designed primers on the flank sequences of each insert (named *inner primers*) as well as on the insert region (named *insert primers*) (See Table 1). Using *inner primers*, the PCR products were differentiated by size since the amplification with insert yielded products of larger size, whereas the amplification without insert yielded products of smaller size. $Ca_v2.3$ containing insert was further verified using *insert primers*, by which we obtained PCR products only in the amplification containing insert, but no products in that lacking insert. 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 is 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

We adopted methods described by Silbert et al (Silbert *et al.*, 2003) for single-cell RT-PCR. The entire single cell was aspirated into a patch pipette under visual control via negative pressure. Patch 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 I was performed before RT. RT was carried out for 1 h at 50°C (Invitrogen) and the cDNA product was used in separate PCR. All PCR amplifications were performed using nested primers as described in Table 1. The forward and reward 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

Table 1. List of primer sequences designed for this study.

Primers	Size (bp)		Primer sequence	Length (bp)
$Ca_v2.3$ Insert I (57bp)	Outer[1] 501/444*	Forward (1751-1770)	ATG GGA CTC CTT CGG CTA AT	20
		Reverse (2232-2251)	CTT TGT TGA GGG CTT CTT GG	20
	Inner[2] 260/203	Forward (1992-2011)	CAA GGA TGA GCA GGA GGA AG	20
		Reverse (2232-2251)	CTT TGT TGA GGG CTT CTT GG	20
	Insert[3] 378/ X	Forward (1751-1770)	ATG GGA CTC CTT CGG CTA AT	20
		Reverse (2108-2129)	CAC ATC GAC ATG TGG TGT CTT C	22
$Ca_v2.3$ Insert II (21bp)	Outer 516/495	Forward (2305-2324)	AAC CCA CTC AAT GCT CAT CC	20
		Reverse (2800-2820)	CAT CAG ACT GTT GGT CCT CCT	21
	Inner 280/259	Forward (2305-2324)	AAC CCA CTC AAT GCT CAT CC	20
		Reverse (2564-2584)	GGT CCT CAA AAG TCA CCA CAG	21
	Insert 392/ X	Forward (2429-2448)	ACA TAG GTG GCC TGA CTA GC	20
		Reverse (2800-2820)	CAT CAG ACT GTT GGT CCT CCT	21
$Ca_v2.3$ Insert III (129bp)	Outer 520/391	Forward (5659-5678)	TCC TCA CTG CCT CAG GAG AT	20
		Reverse (6159-6178)	AGC GGT GAG TGT CAG ACT TG	20
	Inner 267/138	Forward (5738-5757)	GTC GGA GTG GAT ACC CTT CA	20
		Reverse (5985-6004)	AAC GTC GCA TAG AGC TAG GG	20
	Insert 307/ X	Forward (5738-5757)	TCC TCA CTG CCT CAG GAG AT	20
		Reverse (5946-5965)	CCA TAG TCA GAC GTG GCA TA	20
β-actin NM_031144	Outer 456	Forward	CCC AGA TCA TGT TTG AGA CC	20
		Reverse	AGG ATT CCA TAC CCA GGA AG	20
	Inner 317	Forward	AGG CTG TGT TGT CCC TGT AT	20
		Reverse	CAG CTC ATA GCT CTT CTC CA	20
TRPV1 AF029310	Outer 426	Forward	ATG CCA GCT ACA CAG ACA GCT A	22
		Reverse	CCT TCC TGT TGG TGA TCT CTT C	22
	Inner 245	Forward	TGA CCC TCT TGG TGG AGA AT	20
		Reverse	TGT GTT ATC TGC CAC CTC CA	20

outer[1]: *outer primers* used for single cell RT-PCR
inner[2]: *inner primers* used for both whole tissue and single cell RT-PCR
insert[3]: *insert primers* used for both whole tissue and single cell RT-PCR
n/n*: indicates product size which contains insert or lacks insert, respectively

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. For the positive controls, β-actin primers were used in parallel with each amplification. The sequences of all the primers used for single-cell PCR were presented in Table 1. 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).

Results

Two $Ca_v2.3$ isoforms, $Ca_v2.3a$ and $Ca_v2.3e$, are expressed in TG neurons.

The presence of three major inserts were examined to determine structural variants of $Ca_v2.3$; insert I and insert II in the II-III loop, and insert III in the carboxy terminus (Fig. 1). We found that, for the II-III loop, the amplification yielded $Ca_v2.3$ isoforms only lacking insert I and containing insert II. However, for the carboxy terminus, we obtained both shorter cDNA fragment which lacks insert III and longer cDNA fragment which contains the 129-bp fragment

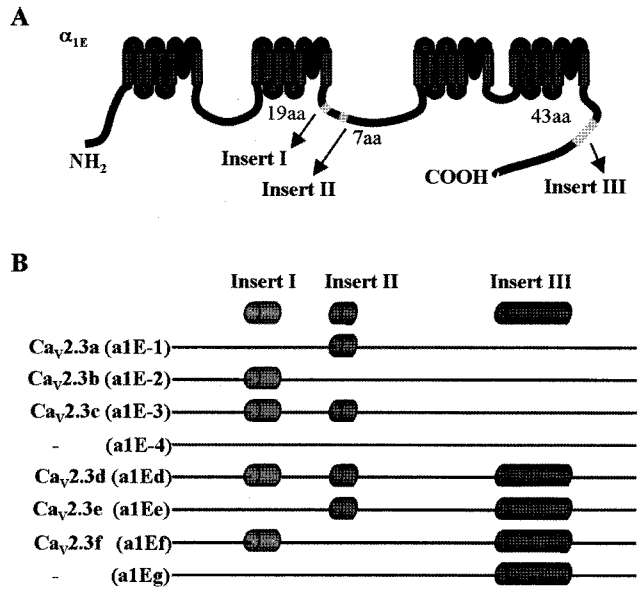

Fig. 1. A, Putative membrane topology of $Ca_V2.3$ subunit. The structural variations cover two segments of 19 (insert I) and 7 amino acids (insert II) in the loop between domain II and III, and a third segment of 43 amino acids (insert III), in the proximal carboxy terminus. **B,** Eight possible isoforms which can be deduced from the $Ca_V2.3$ sequence. $Ca_V2.3a$-$Ca_V2.3f$ is newly proposed names by Pereverzev *et al.* (2002), and the isoform names in parenthesis are initial names.

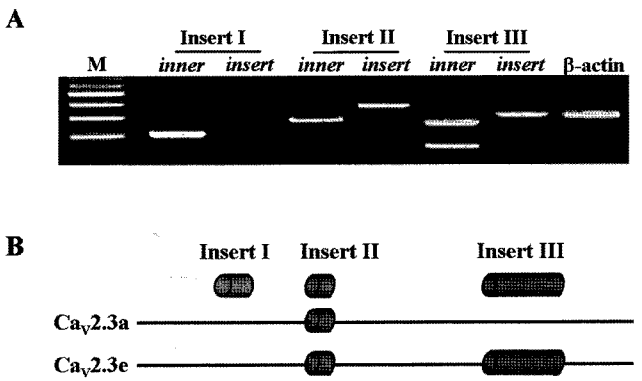

Fig. 2. Two $Ca_V2.3$ isoforms were detected in trigeminal ganglion (TG) neurons. **A,** Insert I, II and III was analyzed by whole tissue RT-PCR. *Inner primers* were designed on the flank of each insert and *insert primers* were designed on the insert region of each insert. Thus, $Ca_V2.3$ isoforms missing insert should generate smaller PCR product with *inner primer* and no product with *insert primer* via RT-PCR. In contrast, $Ca_V2.3$ isoforms containing insert will generate larger PCR products with *inner primer* and product of expected size with *insert primer*. The expected products size is as follows. *Inner primers*: insert I (203 bp) and +insert I (260 bp); insert II (259 bp) and +insert II (280 bp); insert III (138 bp) and +insert III (267 bp); *Insert primers*: insert I (no product) and +insert I (378 bp); insert II (no product) and +insert II (392 bp); insert III (no product) and +insert III (307 bp). $Ca_V2.3$ isoforms amplified from the TG neurons have insert II, but not insert I. regarding insert III, it either lacks or contains insert III. **B,** $Ca_V2.3$ isoform which has insert II, but not insert I or III, corresponds to $Ca_V2.3a$. $Ca_V2.3$ isoform which has insert II and III, but not insert I, corresponds to $Ca_V2.3e$.

encoding insert III. These two isoforms corresponded to $Ca_V2.3a$ and $Ca_V2.3e$, respectively (Fig. 2).

$Ca_V2.3e$ is the major $Ca_V2.3$ isoform in TG neurons.

From whole tissue RT-PCR analyses, we found that $Ca_V2.3a$ and $Ca_V2.3e$ isoforms are expressed in TGs. We then determined the exact pattern of these two isoforms at single cell levels. The patterns of alternative splicing obtained from single-cell PCR were the same as those obtained in the whole tissue, detecting only two $Ca_V2.3$ isoforms, $Ca_V2.3a$ and $Ca_V2.3e$ (Fig. 3A). Therefore, we only analyzed insert III to examine expression pattern of these two $Ca_V2.3$ isoforms in individual neurons. While in one group where nested primers were designed on flank of insert III, this set yielded product of 267 or 138 bp, in the other group where nested primers were designed within target region of insert III, this set yielded product of 307 bp or no product, depending on the presence of insert III. From 78 neurons (chosen irrespective of size), $Ca_V2.3e$ and $Ca_V2.3a$ mRNAs was found in 19.2% and 2.5% of neurons analyzed, respectively (Fig. 3B). We did not observe any sensory neuron that expresses both $Ca_V2.3e$ and $Ca_V2.3a$ in the same cells. Our data show that $Ca_V2.3e$ is the predominant isoform of $Ca_V2.3$ in rat TG neurons.

Expression of $Ca_V2.3e$ isoform is predominant in small-sized and TRPV1-positive TG neurons.

The observation that $Ca_V2.3e$ is the major isoform in TG neurons led us to only analyze this isoform in the subsequent single-cell RT-PCR reaction. Neonatal TG neurons isolated from rat was 10-30 μm in diameter, and categorized into small-sized (10~16 μm), medium-sized (16~20 μm) and large-sized (20~30 μm) neurons, as previously described (Murray *et al.*, 2003). $Ca_V2.3e$ mRNA were detected in 30.9% (n=13/42) of small-sized neurons, 11.1% (n=2/18) of medium-sized neurons, and 0% (n=0/18) of large-sized neurons (Fig. 4A). We further examined whether $Ca_V2.3e$ mRNAs are expressed along with TRPV1 mRNAs in small neurons. 57.1% (n=24/42) of small neurons expressed TRPV1 mRNA, and 76.9% (n=10/13) of neurons that expresses $Ca_V2.3e$ were TRPV1-positive (Fig. 4B).

Discussion

R-type calcium channel has been demonstrated to play a critical role in pain transmission in sensory neurons. In the present study, we identified isoforms of the $Ca_V2.3$ subunit expressed in sensory neurons, located in trigeminal ganglia. Although $Ca_V2.3a$ and $Ca_V2.3e$ were found in TG neurons, $Ca_V2.3e$ was the major one. $Ca_V2.3e$ isoform is preferentially expressed in small-sized TRPV1-positive nociceptive neurons.

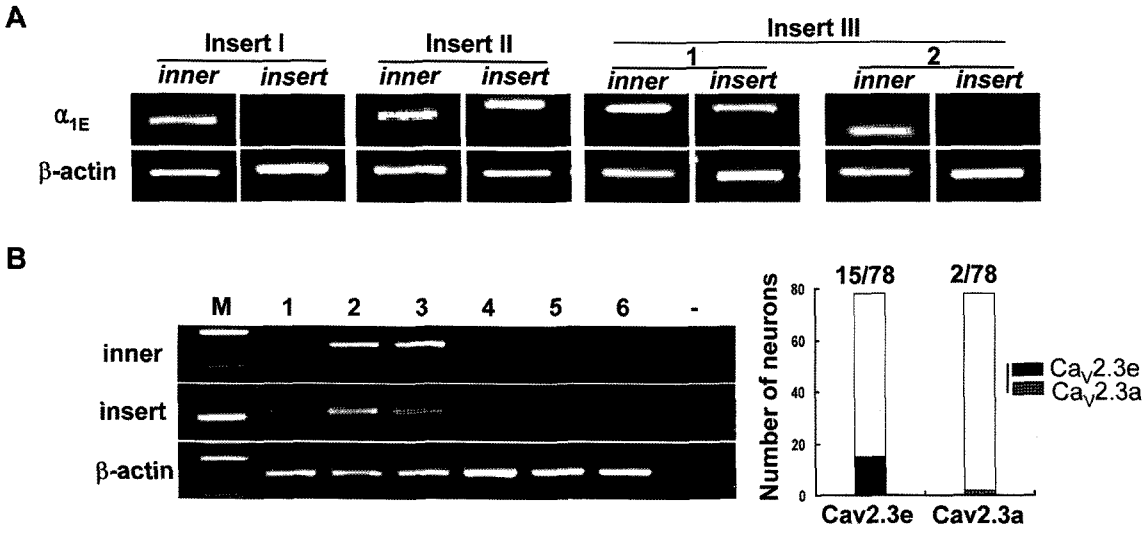

Fig. 3. Demonstration of $Ca_v2.3a$ and $Ca_v2.3e$ isoform from individual TG neurons. **A,** Single-cell RT-PCR products amplified with nested primer from individual neuron were the same as PCR products from whole tissue. i.e. missing insert I, containing insert II and either containing (1) or missing (2) insert III. Note that two PCR products, with or without insert III, (larger, 1 and smaller, 2) were only produced in different cells at single-cell level, indicating that $Ca_v2.3a$ and $Ca_v2.3e$ were not expressed together in the same cells. **B,** Representative gels showing single-cell RT-PCR products amplified using insert III-specific primers in TG. The number (1-6) indicates six different neurons examined from single-cell RT-PCR reaction. β-actin was used in each reaction as a positive control. Bar graph shows percentage of TG neurons that contain $Ca_v2.3e$ and $Ca_v2.3a$, respectively. These results show that $Ca_v2.3e$ is the major $Ca_v2.3$ isoform in TG neurons.

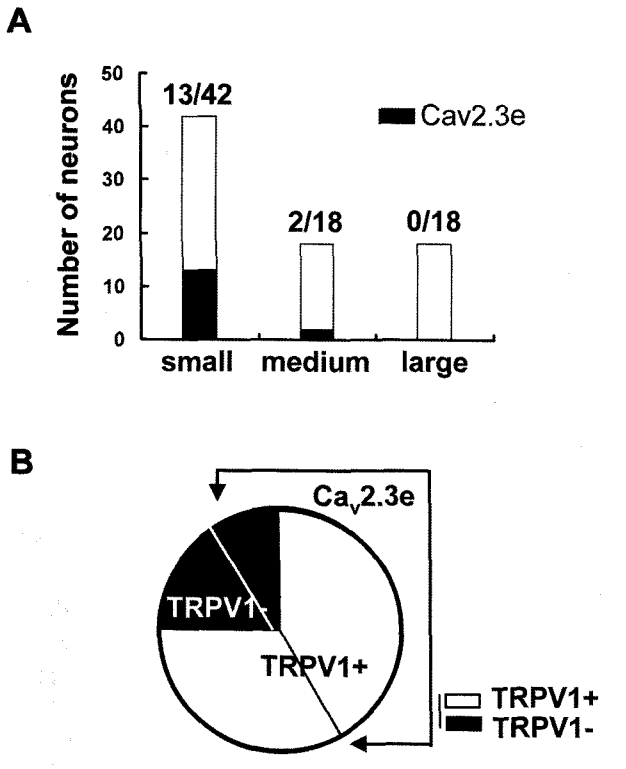

Fig. 4. Distribution of $Ca_v2.3e$ in TG neurons. **A,** Expression pattern of $Ca_v2.3e$ was analyzed in three groups categorized based on cell size (small, medium and large) in TG neuorns. The numbers of $Ca_v2.3e$-expressing neurons were presented in bar graph. These results show that $Ca_v2.3e$ is preferentially expressed in small-sized neurons. **B,** Graph shows that $Ca_v2.3e$ is preferentially expressed in TRPV1-positive neurons.

R-type currents which may result from $Ca_v2.3$ are found in most neurons, such as neocortical and striatal neurons (Foehring *et al.*, 2000), CA1 neurons (Ishibashi *et al.*, 1995), dentate granule cells, cerebellar granule neurons (Schramm *et al.*, 1999) and sensory neurons such as TG neurons (Ikeda *et al.*, 2003). The $Ca_v2.3$ transcripts are widely expressed throughout the brain, as shown by *in situ* hybridization techniques (Niidome *et al.*, 1992). The full-length cDNA from human $Ca_v2.3d$ with a genomic fragment from the human genome (NT_004552.7) revealed the intron-exon structure of the $Ca_v2.3$ gene, which is composed of 49 exons including a 58-bp 3' non-coding segment. Exon 19 encodes insert I of the II-III loop and exon 45 encodes insert III of the carboxy-terminal region, and 21-bp insert II is located within exon 20. Six isoforms ($Ca_v2.3a$, $Ca_v2.3b$, $Ca_v2.3c$, $Ca_v2.3d$, $Ca_v2.3e$, $Ca_v2.3f$) have been reported in mammalian tissues, with the neuronal $Ca_v2.3c$ and the endocrine $Ca_v2.3e$ being the predominant isoforms detected *in vivo*. While $Ca_v2.3a$ has been detected in rat cerebellar granule cells (Schramm *et al.*, 1999), $Ca_v2.3e$ was initially identified in rat and human kidney, insulinoma cell line INS-1 cells and islets of Langerhans (Vajna *et al.*, 1998). We found that TG neurons express two $Ca_v2.3$ isoforms, $Ca_v2.3a$ and $Ca_v2.3e$, both of which contain insert II, but lack insert I, and either lacks or contains exon 45-encoded insert III, respectively. Of these two isoforms, $Ca_v2.3e$, which is known to be major a endocrine $Ca_v2.3$ isoform (Vajna *et al.*, 1998), was the predominant isoform in TG neurons. It is not clear at this moment how $Ca_v2.3e$ contributes to the regulation of neurotransmitter release such as substance P and calcitonin

gene-related peptide from nociceptive nerve terminals as well as insulin secretion from islets of Langerhans. However, it is possible that $Ca_v2.3e$ might be an important molecular mediator which is involved in both neurotransmitter release and hormone secretion. Several previous studies support that R-type calcium channel is crucial for nociception. R-type calcium channel is located at primary synapses (Brown *et al.*, 2004) and contributes to neurotransmitter release and presynaptic plasticity (Dietrich *et al.*, 2003). α_{1E}-/- mice showed reduced response to somatic inflammatory pain (Saegusa *et al.*, 2000). Based on our results, $Ca_v2.3e$ might be the $Ca_v2.3$ isoform responsible for the nociception mediated by $Ca_v2.3$. Therefore, a drug that specifically targets the $Ca_v2.3e$ isoform in nociceptive neurons would be potential target for the treatment of pain.

Sensory neurons which contain $Ca_v2.3e$ exhibited several principle characteristics of nociceptors as $Ca_v2.3e$ was preferentially present in small-diameter TRPV1-positive neurons in the rat TG. Small-diameter neurons and associated Aδ- and C-fiber afferents are generally considered as nociceptors since they are capable of detecting noxious stimuli and initiating pain sensation (Hunt *et al.*, 2001). In response to noxious stimuli, these putative nociceptive neurons generate action potentials, which trigger neurotransmitter release from presynaptic afferent terminals to second-order neurons in the spinal cord. Because we found that $Ca_v2.3e$ is the major isoform in small neurons, $Ca_v2.3e$ is likely to play pivotal role in the synaptic transmission between presynaptic terminals of nociceptive neurons and the second-order neurons. Previous studies have reported using immunohistochemistry (Murakami *et al.*, 2001) and *in situ* hybridization (Yusaf *et al.*, 2001) that α_{1E} subunits were heterogeneously localized in the cell bodies of the DRG neurons. $Ca_v2.3e$ expression at a higher level in the small-sized neurons suggests that $Ca_v2.3e$ might have distinctive role in transduction of pain in nociceptive neurons. TRPV1 has been widely used as a nociceptor marker as it is preferentially expressed in subpopulation of small nociceptive sensory neurons and plays an important role in thermal nociception and inflammatory hyperalgesia (Szallasi *et al.*, 1999). Preferential expression of $Ca_v2.3e$ in TRPV1-positive neurons suggests again a potential role of $Ca_v2.3e$ in transmission of pain information in nociceptive neurons. However, the exact functional role of preferential $Ca_v2.3e$ expression in TRPV1-positive neurons remains to be elucidated.

In summary, our study provides the first evidence that two $Ca_v2.3$ calcium channel isoforms are expressed in rat TG neurons; a major isoform ($Ca_v2.3e$) and a minor isoform ($Ca_v2.3a$). The $Ca_v2.3e$ is predominantly detected in small-sized TRPV1-positive neuorns. The expression pattern of $Ca_v2.3$ isoforms suggests that $Ca_v2.3e$ may play important in pain transmission in trigeminal system. $Ca_v2.3e$ in nociceptive neurons may be a potential target for the pain treatment.

Abbreviations

high-voltage-activated calcium channels, HVACCs; Reverse transcription-polymerase chain reaction, RT-PCR; trigeminal ganglion, TG; transient receptor potential vanilloid 1, TRPV1; voltage-activated calcium channels, VACCs

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