Molecular cloning and tissue distribution of an alternatively spliced variant of an A-type K^+ channel α-subunit, Kv4.3 in the rat

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Abstract **We describe here (1) the heterogeneous expression of Ca^{2+}-independent transient (A-type) K^+ channel α-subunits (Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3) in rat smooth muscle, heart and brain, (2) the molecular cloning and tissue distribution of a novel alternatively spliced variant of an A-type K^+ channel α-subunit, Kv4.3, and (3) the functional expression of A-type K^+ channels in HEK293 cells by the transfection with the novel splice variant of Kv4.3. A cDNA encoding this splice variant was identified from rat vas deferens by RT-PCR cloning. This cDNA clone contains a 1965 bp open reading frame that encodes for a protein of 655 amino acids. It has a 19 amino acid insertion in comparison with Kv4.3 previously reported in rat brain. RT-PCR analyses showed that the mRNAs of this longer variant are abundantly expressed in a number of smooth muscles of the rat, and that the mRNAs of the previously reported clones are absent. The longer splice variant is very weakly expressed in brain, but is the major product in heart.**

Key words: A-type K^+ channel; Kv4.3 splice variant; Smooth muscle; Vas deferens; RT-PCR cloning; Tissue distribution

1. Introduction

A Ca^{2+}-independent transient or A-type K^+ current (I_t or I_A) has been identified in a wide variety of excitable cells [1]. In nerve, I_A reduces the rate of depolarization following an afterhyperpolarization, and/or contributes to early repolarization of action potential. I_A is thus important in the control of action potential threshold, frequency and duration [2]. I_A has also been identified in several smooth muscle cells including portal vein, ureter, colon, mesenteric artery and vas deferens [3–6]. Several A-type K^+ channel α-subunits have been cloned from mammalian brain (e.g. Kv1.4, Kv3.3, Kv3.4, Kv4.1, Kv4.2 [7,8] and Kv4.3 [9,10]). Recently, on the basis of molecular biological analyses, it has been suggested that the Kv4 family (Kv4.1, Kv4.2 and/or Kv4.3) contributes to the Ca^{2+}-independent transient outward K^+ current in brain and heart [11–14]. These results revealed tissue specific distribution of A-type K^+ channel α-subunits and provided insights into the molecular basis of these K^+ channels. Although the corresponding macroscopic currents can be separated by electrophysiological or pharmacological methods, the molecular identity of I_A in smooth muscles has not been reported previously.

In the present study, we describe diverse distribution of the A-type K^+ channel α-subunits, Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3 in brain, heart and vas deferens. Based on these RT-PCR results, we suggest that Kv4.2 and Kv4.3 but not Kv1.4, Kv3.3 or Kv3.4, contribute to I_A in vas deferens. When cDNAs encoding of A-type K^+ channel α-subunits, Kv4.2 and Kv4.3, were cloned from rat vas deferens, a novel alternatively spliced variant of Kv4.3 was detected.

2. Materials and methods

2.1. Preparation of RNA and reverse transcription

Total RNAs were extracted from homogenates of brain, heart and selected smooth muscles of 5–6 week-old male Wistar rat, using the acid guanidium thiocyanate-phenol method, following digestion with RNase-free DNase. Reverse transcription (RT) was performed according to Gibco BRL's protocol. Total RNA (2 μg) and 200 ng of random hexamer in RNase-free H_2O were heated for 10 min at 70°C and incubated for 10 min at 25°C for annealing. This sample was incubated for 60 min at 42°C using 150 units of SUPERSCRIPT II RNase H^- reverse transcriptase (Gibco BRL) in a solution of a final volume of 20 μl which contained 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, and 1 mM individual dNTPs. After this RT procedure, the reaction mixture was added to 80 μl of distilled water and was used for PCR.

2.2. Expression of A-type K^+ channels (Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3)

The primer sequences were chosen based on the cDNA of Kv1.4 (rat brain, a GenBank sequence number of X16002), Kv3.3 (rat pheochromocytoma, M84210), Kv3.4 (rat brain, X62841), Kv4.2 (rat brain, S64320), Kv4.3 (rat brain, U42975), or glyceroaldehyde-3′-phosphate dehydrogenase (GAPDH) (rat various tissues, X00972). These included:

Kv1.4 (corresponding to nucleotides 834–1079; 246 bp)

- (+): 5′-GAGAGAAGAGGAGGACAGGGCT-3′,
- (−): 5′-TGGGGTGCCGAGGTGTCATTCA-3′.

Kv3.3 (corresponding to nucleotides 259–460; 202 bp)

- (+): 5′-GGCGACAGCGGTAAGATCGTG-3′,
- (−): 5′-GGTAGTAGTTGAGCACGTAGGCGA-3′.

Kv3.4 (corresponding to nucleotides 537–738; 202 bp)

- (+): 5′-CAGGCAGAAGGTGGTAATGGAG-3′,
- (−): 5′-AGACGATGAGCGGGAGTTGG-3′.

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Kv4.2 (corresponding to nucleotides 163–363; 201 bp)

- (+): 5′-CTCTGCCCACCATGACTGCTA-3′,
- (−): 5′-CCACTGCATACCTTTCCCCACAA-3′.

Kv4.3 (corresponding to nucleotides 384–653; 270 bp)

- (+): 5′-CTCCCTCAGCTTCCGCCAGACC-3′,
- (−): 5′-CTGCTGGGTGCCGCGAAGAGTC-3′.

GAPDH (corresponding to nucleotides 532–682; 151 bp)

- (+): 5′-GCCATCACTGCCACTCA-3′,
- (−): 5′-CAGTGAGCTTCCCGTTC-3′.

The RT reaction product (1 μl) was amplified using GeneAmp 2400 (Perkin Elmer) in a total volume of 10 μl of a solution containing 250 mM dNTPs, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 100 ng (Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3) or 25 ng (GAPDH) sense and antisense primers, and 0.25 unit Taq DNA polymerase (Takara). The thermal cycler program used for PCR amplification included: a 0.5 min denaturation step at 94°C, a 0.5 min annealing step at 55°C, and a 0.5 min primer extension step at 72°C. The amplification was performed for 32 cycles. Thereafter, reaction mixtures were heated at 72°C for 7 min. Amplified products were separated on 8 M urea-denatured 5% polyacrylamide gels in Tris-borate/EDTA buffer at 300 V for 90 min, visualized with 1 μg/ml ethidium bromide and documented on FAS 1000 (TOYOBO).

2.3. PCR cloning of the Kv4.2 and Kv4.3 cDNA and DNA sequencing

Oligonucleotide primers were arranged as follows: Kv4.2 (+): 5′-CCAC*GAATTCC*CTGTGAGCCGTGTTCCCCAGTG-3′ corresponding to nucleotides 118–97 and (−): 5′-CACC*CTCGAG*ATTCTTTCCATAGTCAGGGCTC-3′ corresponding to nucleotides 1942–1922 of rat brain Kv4.2. Kv4.3 (+): 5′-CCAC*GAATTC*CTGGTGCGTAGTGTATGAGCA-3′ corresponding to nucleotides 48–29 and (−): 5′-TAGG*TCTAGA*GTGGCCACCCACCAACATAC-3′ corresponding to nucleotides 1981–1962 of rat brain Kv4.3. The sequences in italic are *Eco*RI (GAATTC), *Xho*I (CTCGAG) and *Xba*I (TCTAGA) recognition sites which were added to the insert PCR products into vector plasmid DNA, pBluescript SK^+ (Stratagene) in the proper orientation. The thermal cycler program used for PCR amplification included a 0.5 min denaturation step at 94°C, a 0.5 min annealing step at 55°C and a 2 min primer extension step at 72°C for 40 cycles. In PCR cloning experiments, AmpliTaq Gold (Perkin Elmer) was used as a Taq DNA polymerase. Reaction products were separated on 1% agarose gels in Tris-acetate/EDTA buffer and were recovered from gel fragments using GENECLEAN II (Funakoshi). Following this, the cDNAs were ligated into *Eco*RI/*Xho*I or *Eco*RI/*Xba*I recognition sites of pBluescript SK^+, respectively. Cloned cDNAs were sequenced by dideoxy methods using Thermo Sequenase Cycle Sequencing Kit, with DSQ-1000L machine (Shimadzu).

2.4. Cell culture, transfection and electrophysiology

Human embryonal kidney cell line (HEK293) was obtained from Health Science Research Resources Bank (HSRRB) and maintained in Minimum Essential Medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml). A mammalian expression vector, pcDNA3.1 (Invitrogen), was used for stable transfection of HEK239 cells with Kv4.3 by calcium phosphate precipitation and then G418 (Gibco BRL) resistance cells were selected as previously described [15] and identified by Northern blot hybridization.

Electrical recordings of A-type K^+ current were obtained from non-transfected or Kv4.3-transfected HEK293 cells using whole-cell voltage-clamp techniques at room temperature (23 ± 1°C). CEZ-2200 (Nihon Kohden) amplifier was used. The resistance of the recording pipettes ranged from 1–3 mΩ. The series resistance was partly compensated electrically under whole-cell clamp. Data were stored and analyzed in a computer using software, AQ and Cellsoft, which were developed in University of Calgary, Canada. Leakage currents at potentials positive to −80 mV were subtracted on the computer assuming a linear relationship between current and voltage in the range of −120 and −80 mV. The pipette filling solution had the following composition (mM): 140 KCl, 4 $MgCl_2$, 5 Na_2ATP, 0.05 EGTA, and 10 HEPES. The pH was adjusted to 7.2 with KOH. The external solution had the following composition (mM): 137 NaCl, 5.9 KCl, 2.2 $CaCl_2$, 1.2 $MgCl_2$, 14 glucose, and 10 HEPES. The pH was adjusted to 7.4 with NaOH.

2.5. Determination of Kv4.3 splice variant mRNA levels

Total RNA extraction, RT and PCR reactions were performed as described above. PCR oligonucleotide primers for three splice variants of Kv4.3 were designed, as follows. For Kv4.3S (see Section 3), (+): 5′-AGCACCATCCACATCCAG-3′ corresponding to nucleotides 1663–1680 and (−): 5′-GTGTTGGGTTCGTGGAGA-3′ corresponding to nucleotides 1941–1924 of rat hippocampus Kv4.3 cDNA (a GenBank sequence number of L48619) [16]. For Kv4.3M and Kv4.3L, on the basis of 19 amino acid insertion at C-terminal intracellular region, the primers consist of: (+): 5′-GGCACCCCA-GAAGAGGAGCA-3′ corresponding to nucleotides 1372–1391 and (−): 5′-AGCAGCAGGTGGTGGTGAGG-3′ corresponding to nucleotides 1642–1623 of rat brain Kv4.3 cDNA (U42975). The thermal cycler program used for PCR amplification included: a 0.5 min denaturation step at 94°C, a 0.5 min annealing step at 60°C and a 0.5 min primer extension step at 72°C for 40 or 30 cycles. Amplified products were separated on 8 M urea-denatured 5% polyacrylamide gels, visualized with 1 μg/ml ethidium bromide and documented on FAS 1000. Relative band intensities were quantified by densitometric scanning of the negatives using PDI densitometer (Quantity One).

3. Results

3.1. Diverse distribution of A-type K^+ channel α-subunits

The mRNA species responsible for the A-type K^+ channel α-subunits, namely Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3, were quantitated using ethidium bromide-stained gels of RT-PCR products. The specificity of each amplified product was established from the size of the fragment stained with ethidium bromide and confirmed by sequencing analyses. GAPDH mRNA was used to provide a basis for the comparison of these five mRNA samples (Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3), which are candidates for A-type K^+ channels. The cycle numbers used for amplification, and the amount of each primer were carefully adjusted to avoid saturation during amplification. A linear relationship between the cycle number and the band density was observed between 26 and 40 cycles. Furthermore, a linear relationship between the amount of total RNA and band density was observed within the range of 0.1 to 10 μg of total RNA, suggesting that RT-PCR efficacy is constant under these conditions. For each type of RNA, 246 bp (Kv1.4), 201 bp (Kv4.2), 270 bp (Kv4.3), 151 bp (GAPDH) fragments were easily identified by ethidium bromide staining after a 32 cycle PCR, respectively.

Based on these RT-PCR results, a heterogenous distribution of A-type K^+ channel α-subunits was identified in brain, heart and vas deferens (Fig. 1). Although Kv4.2 and Kv4.3 mRNAs were detected in all three tissues, Kv1.4 mRNA could not be detected in vas deferens (Fig. 1A; $n = 6$). Furthermore, both Kv3.3 and Kv3.4 mRNAs were expressed at a relatively high level in brain but were undetectable in heart and vas deferens (Fig. 1B). The amount of Kv4.2 mRNA relative to that of GAPDH was 12.6 ± 1.4, 9.3 ± 1.0 and 9.4 ± 0.4% ($n = 6$) in brain, heart and vas deferens, respectively. The relative amount of Kv4.3 mRNA was 43.5 ± 2.0, 8.9 ± 0.4 and 51.0 ± 2.6% ($n = 6$) in brain, heart and vas deferens, respectively.

3.2. RT-PCR based cloning of Kv4.2 and Kv4.3 in rat vas deferens

Full length Kv4.2 and Kv4.3 clones from vas deferens were PCR amplified with specific primers for each of these K^+ channel isoforms (see Section 2). Lanes 1 and 2 in Fig. 2 show a representative pattern from 1.8% agarose gel electrophoresis of PCR products for Kv4.2 and Kv4.3 mRNA in vas deferens. Amplified products of approximately 2060 bp (Kv4.2) and 2030 bp (Kv4.3) were identified by ethidium bromide staining. Each amplified product was digested with *Eco*RI/*Xho*I (Kv4.2) or *Eco*RI/*Xba*I (Kv4.3) and was ligated into *Eco*RI/*Xho*I or *Eco*RI/*Xba*I double-digested site of pBluescript SKU+, which were termed pBS-Kv4.2 and pBS-Kv4.3, respectively. The fragments arising from *Hin*dIII/*Kpn*I cleavage of pBS-Kv4.2 gave four bands (approximately 350, 800, 850 and 2950 bp) (data not shown) with the predicted sizes for brain Kv4.2 previously reported [8]. The fragments arising from *Eco*RI/*Bam*HI cleavage of pBS-Kv4.3 gave two bands (approximately 1000 bp and 4000 bp) with the predicted sizes for brain Kv4.3 [9]. In contrast, the fragments arising from *Bam*HI/*Xba*I cleavage of pBS-Kv4.3 gave two bands (approximately 1000 and 4000 bp) (lane 4), which are different from the predicted sizes (approximately 950 and 4000 bp). Subsequently, rat brain Kv4.3 was cloned using RT-PCR. The resultant pBS brain Kv4.3 was digested with *Bam*HI/*Xba*I, giving two bands (approximately 950 and 4000 bp) with the predicted sizes (lane 3). These results suggest that a novel splice variant of Kv4.3 may be expressed in vas deferens.

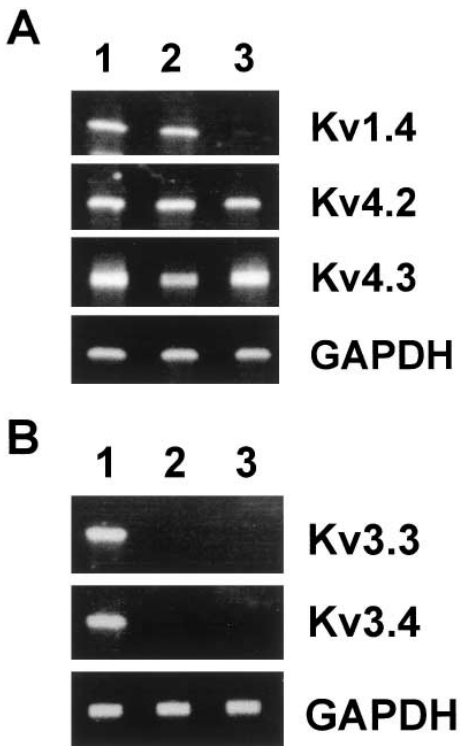

Fig. 1. Diverse distribution of A-type K^+ channel α-subunits (Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3) mRNAs identified by RT-PCR. A: RT-PCRs were performed with four pair primers (Kv1.4, Kv4.2, Kv4.3 and GAPDH) for 32 cycles (see Section 2). B: RT-PCRs were performed with three pair primers (Kv3.3, Kv3.4 and GAPDH) for 32 cycles. cDNA was obtained by reverse transcription of the total RNAs extracted from rat brain (lane 1), heart (lane 2) and vas deferens (lane 3), respectively. Amplified products were separated on 8 M urea-denatured 5% polyacrylamide gels and analyzed by ethidium bromide staining. 246 bp (Kv1.4), 202 bp (Kv3.3), 202 bp (Kv3.4), 201 bp (Kv4.2), 270 bp (Kv4.3) and 151 bp (GAPDH) fragments were identified by ethidium bromide staining, respectively.

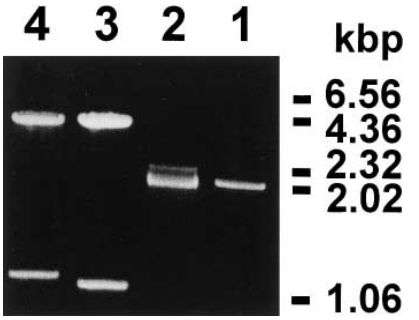

Fig. 2. Analysis of amplified products by 1.8% agarose gel electrophoresis. Lane 1: PCR products from vas deferens for Kv4.2. Lane 2: PCR products for Kv4.3 mRNA. Lane 3: pBS brain Kv4.3 was digested with *Bam*HI/*Xba*I, giving two bands (approximately 950 and 4000 bp) with the predicted sizes. Lane 4: the fragments arising from *Bam*HI/*Xba*I cleavage of pBS-Kv4.3 yielded two bands (approximately 1000 and 4000 bp), which are different from the predicted sizes (approximately 950 and 4000 bp). The migration of the size marker (ϕX 174/Hinc II) is shown on the right.

Nucleotide sequences were determined using dideoxynucleotide chain termination (see Section 2). The sequence of Kv4.2 in vas deferens (vKv4.2) is nearly identical to rat Shal 1 (brain Kv4.2) reported by Baldwin et al. [7], with the exception of one amino acid substitution at residue 537 (Ser-Thr). As reported [7], the amino acid sequences of Shal 1 and Kv4.2 from brain are virtually identical at both the nucleotide and amino acid levels, except for a distinct C-terminus of RK5 (heart Kv4.2) cloned by Roberds et al. [8]. Therefore, RT-PCRs and successive subcloning of Kv4.2 from rat heart and brain cDNA were performed again. The results obtained from 10 independent experiments revealed that both brain and heart Kv4.2 nucleotide sequences are identical to vKv4.2.

As predicted from restriction enzyme mapping (Fig. 2), a splice variant of Kv4.3, which has the insertion of 57 bp in the intracellular C-terminal region, was detected in vas deferens. This region was termed the 'splicing box'. The deduced amino acid sequence of the intracellular C-terminal region in the splice variants of Kv4.3 is shown in Fig. 3. The 'splicing box' contains 19 amino acids, GLSYLVDDPLLSVRTSTIK (residue: 488–506); more than half of which are non-charged residues, and have relatively high hydrophobicity. Database searches (GenBank and SWISS-PROT) yielded no significant homology for this 'splicing box' region with the known other sequences, at either the nucleotide or the amino acid levels. Computer analysis of this clone (GENETYX-MAC) suggests that the 'splicing box' may have a site for protein kinase phosphorylation; specifically that this splice variant of Kv4.3 in vas deferens has a C-terminal domain with additional potential sites for phosphorylation by protein kinase C (Fig. 3).

Recently, another splice variant of Kv4.3, with an open reading frame of 611 amino acids (Kv4.3S) has been isolated from rat hippocampus [16]. The nucleotide sequences are divergent in C-terminus, resulting in a frame shift in the predicted coding sequence. Moreover, in this splice variant, the 'splicing box' is apparently not present. Therefore, specific oligonucleotide primers for Kv4.3S on the basis of C-terminal

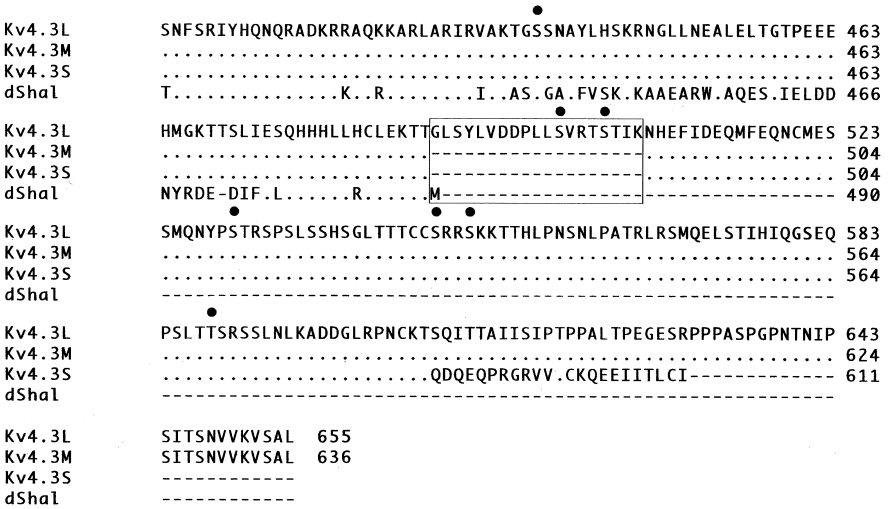

Fig. 3. Alignment of the amino acid sequences of alternatively splice variants of Kv4.3 in intracellular C-terminal regions. The amino acid sequences corresponding to four variants of *Drosophila* Shal (dShal) and Kv4.3 from rat brain (Kv4.3M and Kv4.3S) and vas deferens (Kv4.3L) were aligned. The sequences, Kv4.3M, Kv4.3S and dShal have been reported previously by Serodio et al. (1996) [10], Tsaur et al. (1997) [15] and Wei et al. (1990) [32], respectively. Dashes indicate gaps introduced into the sequence to improve alignment. Dots indicate the sequences identical to Kv4.3L. Putative protein kinase C phosphorylation sites are identified by closed circles. The insertion residues, by alternative splicing, are denoted by the boxed region.

intracellular region were designed and RT-PCR was performed using brain, heart and vas deferens cDNA, respectively. Amplified products were separated on 8 M urea-denatured 5% polyacrylamide gels and visualized with ethidium bromide. RT-PCR analyses by 40 cycle amplification showed that the mRNAs of this splice variant (Kv4.3S) were strongly expressed in rat brain, but only weakly in heart. In vas deferens, no detectable signal was observed (Fig. 4).

3.3. Tissue distribution of mRNA expression encoding Kv4.3 splice variants

PCR oligonucleotide primers were targeted to identify the 'splicing box' in the C-terminal intracellular region. RT-PCR on the total RNA from rat brain, heart and selected smooth muscles gave 214, 271 and 301 bp products, respectively (Fig. 5A) which were subcloned and sequenced. The products 214 and 271 bp corresponded to the equivalent regions of the splice variants of Kv4.3: these were termed Kv4.3M (214 bp) and Kv4.3L (271 bp), respectively. On the other hand, 301 bp products consisted of non-specific sequence. These two splice variants (Kv4.3M and Kv4.3L) were expressed in different levels in brain, heart and smooth muscle tissues. Fig. 5A demonstrates that, unlike brain and heart, only Kv4.3L is expressed in smooth muscle tissues. Furthermore, the same analyses were performed in kidney, liver, lung, skeletal muscle (phrenicopleural fascia), spleen and pancreas. Interestingly, Kv4.3L was expressed abundantly in kidney, and Kv4.3M alone in skeletal muscle and pancreas. In lover, lung and spleen, both variants were weakly expressed (Fig. 5B).

To analyze the relationship between the band density and the amounts of amplified product, each product was diluted in a selected range and separated using 8 M urea-denatured 5% polyacrylamide gels with several lanes demonstrating the chosen dilutions. Densitometric scanning of bands yielded a linear relationship between the band density, and the log of the amount after the dilution (data not shown). Based on this result, we calculated the amount of the amplified product from the band density. The ratio of Kv4.3M and Kv4.3L could then be determined in brain, heart and vas deferens. Although both splice variants were expressed in brain and heart, note that Kv4.3L mRNA is very weakly expressed in brain but is the major product in heart ($3.9 \pm 1.2\%$ and $89.2 \pm 2.8\%$, respectively) (Fig. 5C). It is important to note that only Kv4.3L mRNA was detected in vas deferens and in the other smooth muscles which were examined (aorta, colon, stomach, urinary bladder; Fig. 5A).

3.4. Functional expression of Kv4.3L in HEK239 cells

To examine whether Kv4.3L cDNA encodes A-type K^+ channel, Kv4.3L was expressed in HEK293 cells. Fig. 6A shows typical recordings of membrane currents elicited by depolarization from −80 mV by 10 mV step. Transient outward currents were observed at potentials positive to −40 mV (Fig. 6B). The current reached the peak within 10 ms and decayed to a steady level within 250 ms at +40 mV. The averaged peak amplitude of the current at +40 mV was 4.21 ± 1.22 nA ($n = 5$). On the other hand, in non-transfected

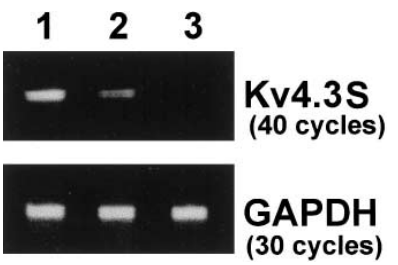

Fig. 4. Expression of a splice variant of Kv4.3S isolated from rat hippocampus [15] with an open reading frame of 611 amino acids. RT-PCRs were performed with a specific degenerate PCR primer for 40 cycles. cDNA was obtained by reverse transcription of the total RNAs extracted from rat brain (lane 1), heart (lane 2) and vas deferens (lane 3), respectively. Amplified products were separated on 8 M urea-denatured 5% polyacrylamide gels. As an internal standard, GAPDH cDNA was amplified for 30 cycles (bottom panel).

cells, more slowly activated and non-inactivated outward currents were elicited by depolarization. The averaged amplitude of outward current at +40 mV was 0.22 ± 0.04 nA ($n = 3$) in non-transfected cells. When 2 mM 4-aminopyridine (4-AP) was applied, the transient outward current activated at +20 mV in a transfected cell was markedly reduced (Fig. 6B, 27.9 ± 2.1% of the control). Similar decrease in the peak outward current by 2 mM 4-AP was observed at any potentials positive to −30 mV (Fig. 6C). These results indicate that A-type K^+ channels sensitive to 4-AP were functionally expressed in HEK293 cells transfected with Kv4.3L.

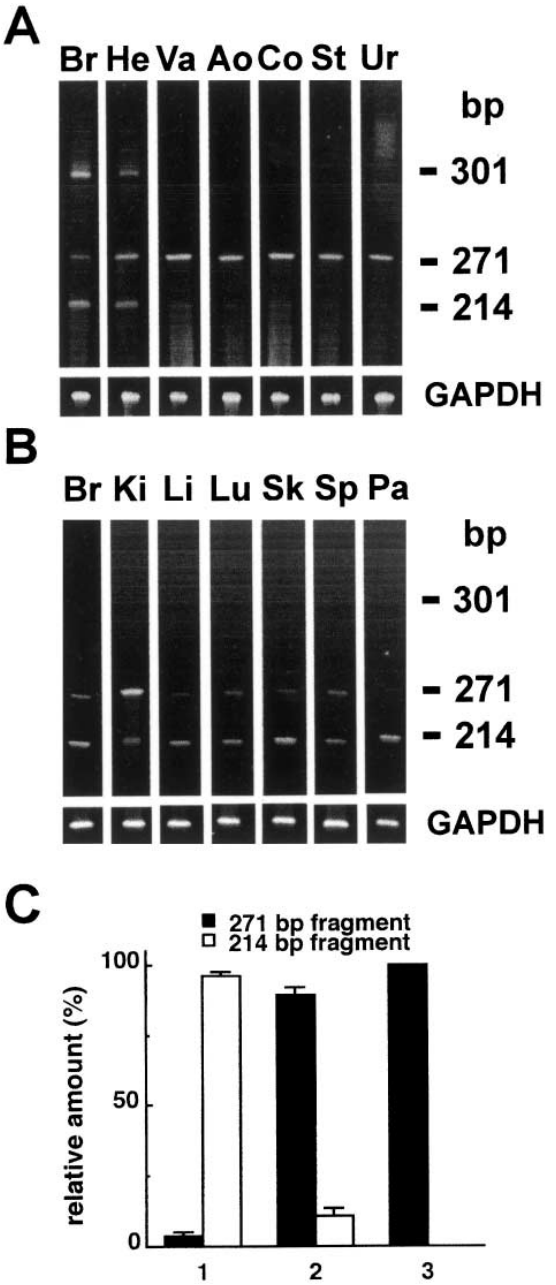

Fig. 5. Tissue distribution of the two splice variants of Kv4.3 by RT-PCR. A: PCR oligonucleotide primers were targeted to identify specific 'splicing box' in C-terminal intracellular region. Amplified products were separated on 8 M urea-denatured 5% polyacrylamide gels, visualized with 1 μg/ml ethidium bromide and quantified by scanning pictures using a PDI densitometer. The sizes of the two PCR products are 214 and 271 bp, respectively. As an internal standard, GAPDH cDNA was also amplified (bottom panel). Lane 1: brain; lane 2: heart; lane 3: vas deferens; lane 4: aorta; lane 5: colon; lane 6: stomach; lane 7: urinary bladder. B: Tissue distribution of the two splice variants was analyzed as described in A. Lane 1: brain; lane 2: kidney; lane 3: liver; lane 4: lung; lane 5: skeletal muscle; lane 6: spleen; lane 7: pancreas. C: Data obtained from separate six experiments in A are summarized. The band densities of amplified fragments were normalized to those of GAPDH, and the relative expression of the splice variants in brain, heart and vas deferens was calculated.

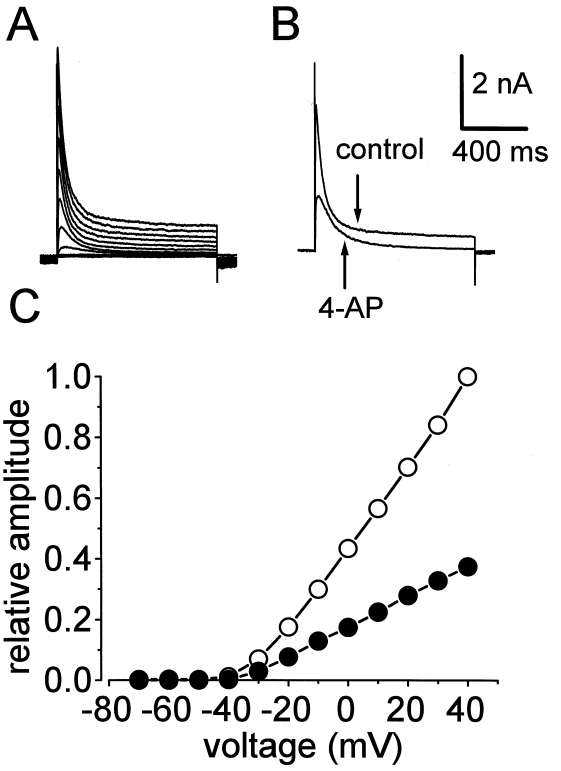

Fig. 6. Membrane currents in HEK293 cells transfected with Kv4.3. A: A transfected cell was depolarized for 1 s from the holding potential of −80 mV to various potentials in a range of −70 and +40 mV by 10 mV set at an interval of 10 s. Transient outward currents were observed at potentials positive to −40 mV. B: Application of 2 mM 4-aminopyridine (4-AP) markedly reduced the transient outward current which was elicited by depolarization from −80 to +20 mV. C: The current-voltage relationships of peak outward current in the absence (○) and presence of 2 mM 4-AP (●). The amplitude at +40 mV was taken as the unity (1.0) in each cell. Each symbol indicates the mean value obtained from 5 cells. The S.E. of the mean was included in the symbols. The averaged peak amplitude at +40 mV was 4.21 ± 1.22 nA and 1.60 ± 0.47 nA in the absence and presence of 4-AP, respectively. The difference is statistically significant when compared with using Student's t-test ($P < 0.01$).

4. Discussion

4.1. Main findings

Our RT-PCR analyses show that, of the K^+ channel isoforms (Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3) which are thought to be responsible for I_A in other tissues [11–14], the mRNA of Kv4.3 is predominant in vas deferens smooth muscle. Moreover, a newly identified splice variant of Kv4.3 is predominant in both cardiac and smooth muscles but is not strongly expressed in brain. In vas deferens, the expression level of Kv4.2 mRNA was only 18% of that of Kv4.3; and Kv1.4, Kv3.3 and Kv3.4 mRNAs were almost undetected. In

heart, as expected from previous reports [9,12], the Kv1.4, Kv4.2 and Kv4.3 mRNAs were present at similar levels.

4-Aminopyridine (4-AP) is used to block I_A in many tissues, including smooth muscles. The Kv3 family of K^+ currents are blocked by 4-AP at millimolar concentrations but are also inhibited by relatively low concentrations (< 5 mM) of externally applied tetraethylammonium (TEA). I_A like currents in cardiac and smooth muscles are not blocked by 10 mM TEA [17,18]. Our findings that Kv3.3 and Kv3.4 mRNAs are absent in heart and vas deferens are, therefore, consistent with this electrophysiological profile.

Although Kv4.2 in rat vas deferens is identical to brain Kv4.2, we identified a novel splice variant of Kv4.3 in vas deferens. It includes an insertion of 57 bp (19 amino acids) in the intracellular C-terminal region (Kv4.3L). The electrophysiological results obtained from HEK293 cells transfected with Kv4.3L clearly showed that Kv4.3L cDNA encodes functional A-type K^+ channel which is sensitive to 4-AP. Interestingly, there are large tissue differences in mRNA expression of three splice variants of Kv4.3 (S, M and L). In smooth and cardiac muscles, Kv4.3L was predominant and the other two variants were virtually absent. In brain, Kv4.3M mRNA was most abundant, although the other two variants were also present at lower levels. It will be interesting to determine whether differences in electrophysiological characteristics can be attributed to these three types of Kv4.3 splice variants. Protein phosphorylation is a key regulatory mechanism of membrane excitability and ion channel function [19]. The fact that the additional 19 amino acid residues in C-terminal region of Kv4.3L include PKC phosphorylation sites may be important in the regulation of the K^+ channels encoded by these splice variants.

4.2. Functional roles of Kv4.3 in smooth muscle cells

Since the resting membrane potential is between -50 and -60 mV in most types of smooth muscles, more than half of I_A channels are inactivated even at rest [3–6]. The major function of I_A in smooth muscle cells is, therefore, regulation of action potential threshold and firing frequency immediately following hyperpolarization, during which a substantial population of I_A channels recovers from inactivation [4]. In contrast, in cardiac myocytes, activation of the Ca^{2+}-independent transient outward K^+ current (I_t) contributes to early phase of action potential repolarization [20]. Kv4.2 and Kv4.3 have been shown to contribute to I_t in rat cardiac myocytes, based on antisense mRNA methods combined with electrophysiological analyses [14].

We have reported that I_A in smooth muscle cells can be blocked by low concentration of arachidonic acid (AA) [6] and that I_t in rabbit atrium is far less sensitive to AA than I_A in rabbit vas deferens [6]. Recently, it has also been shown that AA blocks Kv4 family K^+ channels rather than Kv1, Kv2 and Kv3 families [21]. These findings, in combination with the present results, suggest that I_A in some smooth muscles may mainly be attributable to Kv4.3. The reason why the sensitivity of I_A in ventricular myocytes to AA is much lower than that in smooth muscle is not completely clear in this study. In ventricular myocytes, however, the expression of Kv1.2, Kv1.4, Kv1.5 and Kv2.1 mRNAs are abundant and these themselves or combination with β-subunit may be factors which contribute to I_A [22]. The lower sensitivity of these α-subunits to AA [21] could explain the difference between ventricular and smooth muscle cells.

4.3. Diversity of K^+ channels responsible for I_A

Understanding the diversity of A-type K^+ channels in various tissues is an essential prerequisite to determination of the physiological and/or pharmacological characteristics of these channels and identification of their regulation mechanisms, such as the sensitivity to PKC activators [19], AA [6,23,24] or some K^+ channel blockers [25]. Kv1.4 and Kv4.2 are differentially expressed in rat central neurons, and this pattern of distribution implies distinct roles for these K^+ channel proteins in vivo [26]. Kv4.2 and Kv4.3 have many similarities in structure, and electrophysiological properties; and in some tissues, their subcellular localization is similar. However, these two isoforms have also been suggested to have separate functional roles in neurons which co-express them [16].

Heterotetrametric channel structure, usually involving different Kv families has been suggested as a means of generating channel diversity [27,28]. When hybrid channels consisting of Kv1.4 and other Kv1 family α-subunits are expressed in *Xenopus* oocytes, they exhibit kinetics that more closely resemble a native cardiac I_t [29]. However, since Kv1.4 is absent in vas deferens, it is unlikely that heterotetrametric channels of Kv1 family are mainly responsible for I_A in this tissue.

In addition, channel heterogeneity resulting from alternative splicing of α-subunit has been described [30]. N- and C-terminal are known to play important roles for the formation of homo- and heteromultimers [31,32]. Heteromeric channel complexes consisting of two different splice variants can alter the kinetics of channel re-activation. Indeed, *Drosophila* Shal has much shorter sequences in C-terminal region than rat Kv4.3M and physiologically significant differences in kinetics observed in the currents expressed in *Xenopus* oocytes [10,33]. However, this mechanism appears not to be important in vas deferens smooth muscles, since expression level of Kv4.3L was much higher than those of other variants.

It has been reported that pore-forming α-subunits of some delayed rectifier K^+ channels are converted to A-type K^+ channels by association with peripheral β-subunit [29,34,35]. The possibility that this type of channel complex contributes to I_A in smooth muscle cells can not be ruled out.

In summary, we have identified a heterogeneous pattern of expression of Kv1.4, Kv3.3, Kv3.4, Kv4.2 and Kv4.3 mRNA, and a novel splice variant mRNAs of Kv4.3 in brain, heart and vas deferens. The novel splice variant of Kv4.3 has an additional 19 amino acid residue in cytosolic region near C-terminus, and the corresponding mRNA is predominant in both cardiac and smooth muscles, providing indirect evidence that Kv4.3 contributes to I_A in some smooth muscles. It seems likely that Kv4.3L is probably the K^+ channel responsible for I_A in smooth muscle cells and in cardiac myocytes, even though the functional roles of I_A and I_t in these two types of muscles are not similar.

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