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Expression of skeletal muscle $Na_V1.4$ Na channel isoform in canine cardiac Purkinje myocytes

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Abstract

Background and Aim: The action potential plateau of Purkinje fibers is particularly sensitive to tetrodotoxin (TTX) and this could be due to a TXX-sensitive Na^+ current. The expression of TTX-sensitive neuronal $Na_V1.1$ and $Na_V1.2$ isoforms has been reported in canine Purkinje myocytes. Our aim was to investigate by means of biochemical and functional techniques whether the TTX-sensitive skeletal $Na_V1.4$ isoform is also expressed in canine cardiac Purkinje myocytes.

Methods and Results: Using $Na_V1.4$ specific primers, a PCR product corresponding to $Na_V1.4$ was amplified from canine Purkinje fibers RNA and confirmed by sequencing and megablast of the gene bank. Confocal indirect immunostaining using anti-$Na_V1.4$ antibody demonstrates distinct sarcolemmal staining pattern compared to that of the cardiac isoform $Na_V1.5$. Expression of $Na_V1.4$ in tsA201 cells yielded a TTX-sensitive Na^+ current with an IC_{50} of 10 nM.

Conclusions: These results demonstrate the expression of the TTX-sensitive $Na_V1.4$ channel in canine cardiac Purkinje myocytes. This novel finding suggests a role of $Na_V1.4$ channel in Purkinje myocytes and thus has important clinical implications for the mechanisms and management of ventricular arrhythmias originating in the Purkinje network.
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Keywords: Na^+ channel isoforms; Cardiac tissues; Purkinje myocytes; Tetrodotoxin

The action potential (AP) of Purkinje fibers has a larger upstroke and longer plateau than the action potentials of atrial and ventricular cells [1,2]. The Na^+ channel isoform $Na_V1.5$ underlies the fast-activating and -inactivating Na^+ current and its activation is mostly responsible for the fast and large upstroke of the AP in Purkinje fibres [3]. Therefore, $Na_V1.5$ channel plays a central role in the fast conduction of cardiac impulse in Purkinje fibers [3] and it has a low sensitivity to block by tetrodotoxin (TTX) [4].

In contrast, low concentrations of TTX that have little or no effect on the rate of rise and amplitude of the upstroke substantially shorten the Purkinje fiber AP [4]. Since TTX selectively blocks Na^+ channels [5], the shortening of the plateau with no or small effects on the upstroke suggests that a Na^+ current different from that of $Na_V1.5$ may contribute to the duration of Purkinje fiber plateau. In fact, it has been recently demonstrated that at its threshold (~ -50 mV), Na^+ current quickly activates and inactivates, but in plateau range of potentials a slowly inactivating inward current flows that peaks at -30 to -20 mV. This current is blocked preferentially by TTX and lidocaine [6] and therefore it appears to play a role in the longer plateau of Purkinje fibers.

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These findings raise the question as to which TTX-sensitive Na^+ channels might be responsible for the Purkinje fiber long plateau. So far, nine Na^+ channel α-subunits ($Na_V1.1$ to $Na_V1.9$) distributed in various tissues have been cloned [7,8]. In the heart, different populations of Na^+ channels have been identified which differ in their sensitivity to block by TTX [9]. In the heart, the cardiac Na^+ channels, such as $Na_V1.5$, have a low TTX-sensitivity (they are blocked by micromolar concentration) whereas neuronal $Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.6$, and skeletal muscle $Na_V1.4$ have a high TTX-sensitivity (they are blocked by nanomolar concentrations) [9,10]. In Purkinje myocytes, both TTX-resistant $Na_V1.5$ and TTX-sensitive $Na_V1.1$ and $Na_V1.2$ have been identified [11].

The TTX-sensitive Na^+ channels account for 16% of the channel pool mRNA in single Purkinje adult myocytes, the neuronal Na_V channel isoforms being more abundant in Purkinje than in ventriclular myocytes [10,11]. The expression level of skeletal $Na_V1.4$ is much greater than that of neuronal Na^+ channel isoforms in ventricular myocytes [10]. However, whether or not the skeletal $Na_V1.4$ is expressed in the canine Purkinje myocytes (thereby possibly contributing to the TTX-sensitive Na^+ current) is not known. The aim of this study was to use biochemical, immunohistochemical, and electrophysiological approaches to investigate whether the skeletal muscle $Na_V1.4$ is expressed in canine Purkinje single myocytes. Slowly inactivating plateau currents are a potential source of arrhythmias and the Purkinje system can become the site of origin of ventricular arrhythmias, such as those that occur in the early ischemic period [12,13].

Materials and methods

Total RNA preparation. The animal procedures conformed to the principles embodied in the Declaration of Helsinki, and all procedures related to animal use comply with the *Guiding Principles for Research Involving Animals and Human Beings*. Total cellular RNA was isolated from canine Purkinje tissue using an RNAzol™ B kit [14] as described previously [15,16]. Genomic DNA was removed from all the RNA extract by digestion with DNase. RNA was quantified by spectrophotometry at 260 nm, and the ratio of absorbance at 260 nm to that of 280 nm was >1.8 for all samples. Degradation of RNA samples was monitored by the observation of appropriate 28S to 18S ribosomal RNA ratios as determined by ethidium bromide staining of the agarose gels.

PCR amplification. First-strand cDNA was synthesized with reverse transcriptase using total RNA from canine Purkinje tissue with Retroscript kit from Ambion Inc. [15,16]. The primers for $Na_V1.4$ were selected from the regions that are conserved among different species and unique to $Na_V1.4$ Primer 5′ ccccgagggttatgagtgta, 3′ tctcctgatcctcagccagt. The expected PCR product is 284 bp. For $Na_V1.5$ Primer 5′ atcttctcagcagtgg, 3′ gttgccacctgaagaag, the expected PCR product is 612 bp. An initial denaturation step at 94 °C for 4 min was followed by 36 cycles at 94 °C for 1 min, 53 °C for 1 min and 72° C for 1 min, and a final extensive incubation was done at 72 °C for 10 min. RNA samples without reverse transcriptase were included as control. The PCR products were sequenced commercially.

Isolation of canine Purkinje myocytes. Canine single Purkinje myocytes were isolated as previously described in detail [17,18]. In brief, Purkinje fibers were superfused in the tissue bath at 37 °C and stimulated at 60/min for 30 min. The composition of Tyrode's solution gassed with 100% O_2 was (in mM): NaCl 140, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 5.0, and glucose 5.5 (pH adjusted to 7.4 with NaOH). The fibers were then rinsed with Ca-free solution containing (in mM): NaCl 120, KCl 12, KH_2PO_4 1.2, $MgCl_2$ 1.5, Hepes 5.0, glucose 5.5, taurine 5, β-hydroxybutyric acid 5, and Na pyruvate 5 (pH 7.2). Next, the strips were digested at 37 °C for 40 min in the Ca-free Tyrode solution with collagenase (1 mg/ml, type VIII, Sigma) and bovine serum albumin (2 mg/ml). The final cell suspension was centrifuged and the pellet was re-suspended in a Kraftbrühe (KB) solution (in mM): KCl 85, KH_2PO_4 30, $MgSO_4$ 5.0, glucose 20, pyruvic acid 5.0, creatine 5.0, taurine 5.0, EGTA 0.5, hydroxybutyric acid 5.0, succinic acid 5.0, and Na_2ATP (1.1 mg/ml) (pH 7.2).

Immunostaining. Confocal immunostaining was performed as previously reported [15,16,19]. Single Purkinje myocytes were plated on laminin-coated glass coverslips and fixed with 4% paraformaldehyde for 30 min followed by permeabilization with 0.1% Triton X-100 for 15 min. The fixed cells were quenched for aldehyde groups in 0.75% glycine buffer and blocked with donkey serum and washed with PBS. Cells were incubated overnight with individual antibodies against $Na_V1.4$ and $Na_V1.5$ α1 subunit (Sigma). After extensive wash, the cells were incubated with FITC- or TRITC-conjugated secondary antibodies (Johanson Immunol) for 1 h. To ascertain specificity, cells with the secondary antibody alone were analyzed. Images were acquired with the MRC-600 Bio-Rad confocal microscope.

Transient transfection and recording of Na^+ current from tsA201 cells. TsA201 cells were transfected with cDNA (5 μg) coding for human $Na_V1.4$ and 5 μg of β1 subunit and an expression plasmid for a lymphocyte surface antigen (CD8-a) [21] using the calcium phosphate method [20]. Two to three days post-transfection cells were incubated for 5 min in a medium containing anti-CD8-a coated beads [21] (Dynabeads M-450 CD8-a, Dynal). Cells expressing surface CD8-a fixed the beads and were visually distinguishable from non-transfected cells by light microscopy. Na^+ currents were recorded using the whole-cell patch clamp technique at room temperature [22]. Recordings were made using low resistance pipettes (<1 MΩ), and a routine series resistance compensation by an Axopatch 200 amplifier (Axon Instruments) was performed to values >80% to minimize voltage-clamp errors. Na^+ currents were filtered at 5 kHz, digitized at 10 kHz. Data analysis was performed using pCLAMP v9.0 (Axon Instruments). Patch pipette solution contained (in mM): 35 NaCl, 105 CsF, 10 EGTA, and 10 Cs–Hepes (pH 7.4). The bath solution contained (in mM): NaCl 150, KCl 2, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 10, and Na–Hepes 10 (pH 7.4).

Results

Expression of $Na_V1.4$ mRNA in canine Purkinje fibers

Transcript of $Na_V1.4$ was determined by RT-PCR using total RNA prepared from canine Purkinje fibers using specific $Na_V1.4$ primers. A 284 bp PCR product was amplified. The cardiac $Na_V1.5$ was included as positive control, and showed a 612 bp band. No transcript was identified from sample in the absence of reverse transcriptase, indicating the absence of genomic DNA contamination in the total RNA preparation (Fig. 1). The PCR product using $Na_V1.5$ primers was sequenced and showed 100% homology to the published canine $Na_V1.5$ sequence in gene bank (Fig. 2).

Because the canine $Na_V1.4$ is not cloned yet, the PCR product of $Na_V1.4$ was sequenced and the NCBI *Mega BLAST* program was used to search for sequence homology in the gene bank. $Na_V1.4$, but not other Na_V isoform sequences from different species were pulled out with the highest homology score. The nucleotide sequence of the PCR products using $Na_V1.4$ primers (first row of the sequences in Fig. 2) is 91% identical to human, 93% to horse, and 90% to mouse and cattle $Na_V1.4$ sequences

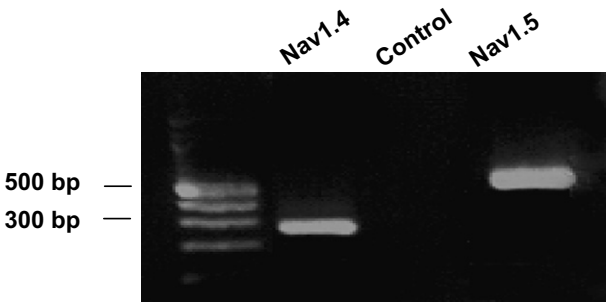

Fig. 1. The mRNA of skeletal muscle isoform $Na_V1.4$ in Purkinje fibers. Transcript of $Na_V1.4$ in canine Purkinje myocytes was determined by RT-PCR using total RNA prepared from canine Purkinje fiber using specific $Na_V1.4$. A 284 bp PCR product was amplified corresponding to $Na_V1.4$. The cardiac $Na_V1.5$, which was included as positive control, showed a 612 bp band. No transcript was amplified from sample in the absence of reverse transcriptase (Control), indicating the absence of genomic DNA contamination in the total RNA preparation.

(Fig. 2). These data indicate the unambiguous expression of $Na_V1.4$ mRNA in canine Purkinje myocytes.

Expression of $Na_V1.4$ protein in canine Purkinje fiber

We next examined the expression and localization of $Na_V1.4$ protein in canine Purkinje fibers by confocal microscopy using isolated cells. As shown in Fig. 3, a clear staining, most prominent on cell sarcolemma, was observed for $Na_V1.4$ (Fig. 3A and B). Interestingly, the staining pattern was in clear contrast to $Na_V1.5$, which showed remarkable striation staining pattern (Fig. 3C and D). No specific staining was observed in cells stained with the secondary antibody alone, indicating the specificity of the antibodies used (Fig. 3E and F). The strikingly different localization of $Na_V1.4$ and $Na_V1.5$ may be related to unique functions of individual Na^+ channel isoforms in Purkinje myocytes.

TTX-sensitivity of $Na_V1.4$ channel

$Na_V1.4$ isoform was co-expressed together with β_1 subunit in tsA201 cells and the TTX sensitivity was tested. Fig. 4A shows representative current traces in control, in the presence of 10, 100 nM TTX and after washout. Fig. 4B illustrates the averaged peak current density during control, and in the presence of 10, 100 nM TTX and washout. The IC_{50} of $Na_V1.4$ current to TTX was 10 nM.

Discussion

In the present study, we demonstrated the expression of the skeletal Na^+ channel isoform $Na_V1.4$ in canine cardiac Purkinje myocytes. Using $Na_V1.4$ specific primers, a PCR product of 284 bp was amplified using total RNA prepared from canine Purkinje fibers. The sequence of the PCR product is highly homologous to $Na_V1.4$, but not to other Na^+ channel isoforms. Expression of the $Na_V1.4$ in tsA201 cells generated a TTX-sensitive Na^+ current with a nanomolar IC_{50}. Furthermore, the existence of $Na_V1.4$ channel protein was confirmed by confocal indirect immunostaining. $Na_V1.4$ showed a distinct pattern of staining when compared to $Na_V1.5$.

Low concentration TTX shorten the plateau of AP in Purkinje fibers with little or no effects on the upstroke [4], suggesting the existence of Na^+ channel isoform that is different from $Na_V1.5$. In a recent study, Vassalle et al.

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Canine Nav1.4       CNATGGCTATACNNGCTATGATACATTCAGCTGGG 35
Human(nm000334.3)   CTATGGCTACACCAGCTATGACACCTTCAGCTGGG 1260
Horse (u25990.1)    CTACGGCTACACCAGTCACGACACCTTCAGCTGGG 1165
Cattle(XM595595.2)CTACGGCTACACCAGCTACGACACCTTCAGCTGGG 1021
Mouse (NM133199.1) CTATGGCTACACCAGCTACGACACCTTCAGCTGGG 1166
                    * * ***** **  *  * ** ** **********

Canine Nav1.4       CCTTCCTGGCTCTCTTCCGCCTCATGACCCAGGACTACTGGGAGAACCTCTTTCAGCTGA 95
Human(nm000334.3)   CCTTCTTGGCTCTCTTCCGCCTCATGACACAGGACTATTGGGAGAACCTCTTCCAGCTGA 1320
Horse(u25990.1)     CCTTCCTGGCTCTCTTCCGCCTCATGATACAGGACTATTGGGAGAACCTCTTCCAGCTGA 1225
Cattle(XM595595.2)CCTTCCTGGCTCTCTTCCGTCTCATGACACAGGACTATTGGGAGAACCTCTTCCAGCTGA 1081
Mouse(NM133199.1)   CCTTCCTGGCTCTCTTCCGTCTCATGACGCAGGACTACTGGGAGAACCTTTTCCAGCTGA 1226
                    ***** ************* *******  ******** *********** ** *******

Canine Nav1.4       CCCTTCGAGCAGCTGGCAAGACCTACATGATCTTCTTTGTGGTCATCATTTTCCTGGGCT 155
Human(nm000334.3)   CCCTTCGAGCAGCTGGCAAGACCTACATGATCTTCTTCGTGGTCATCATCTTCCTGGGCT 1380
Horse(u25990.1)     CCCTTCGAGCAGCCGGCAAGACCTACATGATCTTCTTTGTGGTGATCATTTTCCTGGGCT 1285
Cattle(XM595595.2)CCCTTCGAGCAGCCGGCAAGACCTACATGATCTTTTTCGTGGTCATCATTTTCCTGGGCT 1141
Mouse(NM133199.1)   CCCTTCGAGCTGCTGGCAAGACCTACATGATCTTCTTTGTGGTCATCATCTTCCTGGGTT 1286
                    ********** ** ******************** ** ***** ***** ******** *

Canine Nav1.4       CCTTCTACCTCATTAACCTGATCCTGGCGGTGGTGGCCATGGCGTATGCAGAGCAGAATG 215
Human(nm000334.3)   CTTTCTACCTCATCAATCTGATCCTGGCCGTGGTGGCCATGGCATATGCCGAGCAGAATG 1440
Horse (u25990.1)    CCTTCTACCTCATCAATCTGATCCTAGCAGTGGTAACCATGGCGTACGCGGAGCAGAATG 1345
Cattle(XM595595.2)CCTTCTACCTCATCAACCTGATCCTGGCTGTGGTAGCCATGGCCTACGCTGAGCAGAACG 1201
Mouse(NM133199.1)   CCTTCTACCTCATCAATCTGATCCTGGCGGTGGTGGCCATGGCATATGCTGAGCAGAATG 1346
                    * *********** ** ******** ** *****  ******* ** ** ******** *

Canine Nav1.4       AGGCCACACTGGCTGAGGATCAGGAGAA 243
Human(nm000334.3)   AGGCCACCCTGGCCGAGGATAAGGAGAA 1467
Horse(u25990.1)     AGGCCACCCTGGCTGAGGATCAGGAGAA  1373
Cattle(XM595595.2)AGGCCACCCTGGCCGAGGATCAGGAGAA  1229
Mouse(NM133199.1)   AGGCTACCCTGGCCGAAGACCAGGAGAA 1374
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Fig. 2. $Na_V1.4$ PCR product sequence analysis. The PCR product of $Na_V1.4$ was sequenced and the NCBI Mega BLAST program was used to search for highly similar sequences from Genbank. $Na_V1.4$ sequences from different species were pulled out with the nucleotide sequence 91% identical to human, 93% to horse, and 90% to mouse and cattle $Na_V1.4$ sequences. * indicates the homology.

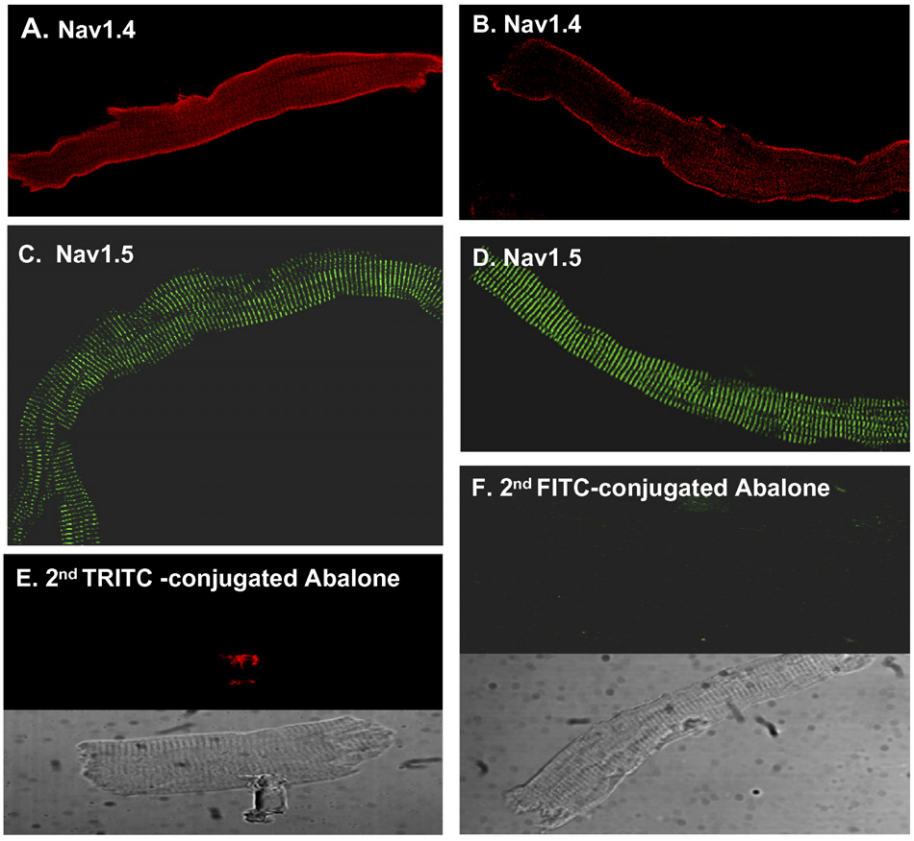

Fig. 3. Expression of skeletal muscle isoform $Na_V1.4$ and cardiac isoform $Na_V1.5$ in Purkinje myocytes: Confocal indirect immunostaining was used to assess the expression and subcellular localization of $Na_V1.4$ on isolated canine Purkinje myocytes. The anti-$Na_V1.4$ antibody (Ab) showed predominant localization on the surface sarcolemma in two different cells (A and B). Unlike $Na_V1.4$, anti-$Na_V1.5$ antibody showed regularly spaced transverse striations across the length of the cells (C and D). $Na_V1.5$, the cardiac Na^+ channel isoform was included as positive control. Secondary antibodies alone (E and F) did not show any specific staining indicating the specificity of the pattern observed in (A–D).

[6] identified a novel slowly decaying inward current (labelled I_{Na2}) that has a less negative threshold, slower inactivation kinetics, and greater sensitivity to TTX than the fast activating and inactivating I_{Na} (labelled I_{Na1}). During depolarizing steps to −50 mV, a large (>10 nA) I_{Na1} rapidly activates and inactivates in a few milliseconds to a steady current. On depolarization to less negative values, the fast decay of I_{Na1} is interrupted by the appearance of I_{Na2} as a slowly decaying inward tail that reaches its largest size at −30 to −20 mV. Because of its slow inactivation in the voltage range of the plateau of Purkinje fibers, I_{Na2} is bound to contribute to the longer plateau in this tissue.

During ramps of different slopes, after the inactivation of I_{Na1}, the slow inactivation of I_{Na2} creates a negative slope in the current–voltage relation. With faster ramps (250 and 125 ms), 30 μM TTX reduced the amplitude of I_{Na2} more than that of I_{Na1}, indicating that I_{Na2} is more sensitive than I_{Na1} to the inhibitory effect of TTX. The greater sensitivity of I_{Na2} to TTX relative to I_{Na1} may account for the fact that in Purkinje fibers TTX at suitable concentrations shortens the plateau [1,4] more than it decreases the amplitude and the rate of rise of the upstroke [4]. In the present experiments, TTX concentration was lower (10 and 100 nA) and the results clearly show that $Na_V1.4$ current is markedly sensitive to TTX.

The protocol adopted demonstrated the high TTX-sensitivity of the $Na_V1.4$ current. As for fast versus slow inactivation, the former occurs within milliseconds [23] whereas the latter develops in seconds [24,25]. The Na^+ channels slow inactivation has been separated from fast inactivation in several ways such as using enzymatic methods, mutating the IFM motif in the III–IV linker, or by adopting pulse protocols that allow fast inactivation to recover before assessing the slow inactivating channels [26]. In the approach of Vilin et al. [26], first both fast and slow inactivation were eliminated by clamping the membrane at −150 mV for 30 s. Gradually longer prepulses up to 0 mV were applied to induce increasing degrees of slow inactivation. A brief (20-ms) pulse was applied to −150 mV to selectively remove the inactivation of the fast channels before a test pulse to 0 mV. The normalized amplitudes of Na^+ currents during the test pulse at 0 mV were then plotted versus the prepulse duration to measure the degree of slow inactivation. Recovery from slow inactivation was studied after the slow channels were inactivated by 60-s prepulse to 0 mV.

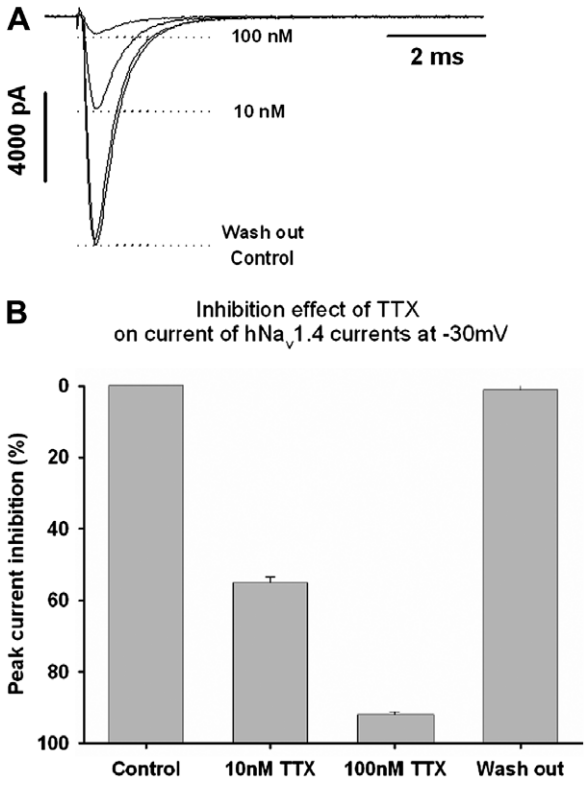

Fig. 4. TTX-sensitivity of expressed human $Na_V1.4$ in tsA201 cells. Peak currents- were evoked at −10 mV for 40 ms every 5 s from a holding potential of −100 mV. The results were generated from five sets of 4–7 individual experiments, and the data are composed of a collective response. The maximum decrease from the baseline was normalized and the pooled data were plotted as the percentage change from the baseline versus the concentration tested. (A) Representative current traces in $Na_V1.4$-β_1 transfected tsA201 cells at basal, 10, 100 nM TTX and washout conditions. (B) Summary of expressed $Na_V1.4$ current at basal, 10, 100 nM TTX and washout conditions.

The differences between I_{Na2} and I_{Na1} elicit the question whether I_{Na2} might be related to a specific Na^+ channel isoform that exhibit slow inactivation. Of the nine different Na^+ channels ($Na_V1.1$ to $Na_V1.9$) identified and functionally expressed [8], in cardiac tissues three kinds of Na^+ channels (cardiac, neuronal and skeletal) have different inactivation kinetics and sensitivity to TTX.

The cardiac isoforms display low TTX-sensitivity and fast inactivation, the $Na_V1.5$ channel generating the bulk of I_{Na1}. The neuronal Na^+ channel isoforms ($Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.6$ and $Na_V1.7$) as well as the skeletal muscle isoform ($Na_V1.4$) [8] are more sensitive to TTX. In this regard, Haufe et al. showed that the proteins of neuronal $Na_V1.1$ and $Na_V1.2$ channels (but not $Na_V1.3$ and $Na_V1.6$) are expressed in canine Purkinje tissue [11]. Also, the neuronal Na^+ channels contribute more to the peak Na^+ current in canine Purkinje than in ventricular cells ($22 \pm 5\%$ vs. $10 \pm 5\%$) [11].

These characteristics raise the question as to whether $Na_V1.4$ might be among the possible Na^+ channels that underlie I_{Na2}. The prerequisite for such a possibility (the expression of $Na_V1.4$ in Purkinje fibers) is fulfilled by the present study, which identified the presence and distinct subcellular localization of $Na_V1.4$ in Purkinje myocytes.

Both veratridine [27] and sea anemone toxin, ATX II [28] delay $Na_V1.4$ inactivation and prolong the AP of Purkinje fibers. $Na_V1.4$ is blocked by TTX, local anesthetics and lidocaine [29], all of which also shortens and shift to more negative values the plateau of Purkinje fibers [1,2,30]. Altogether, these reports indirectly suggest (but do not prove) a potential contribution of skeletal muscle $Na_V1.4$ isoform to the AP of Purkinje fibers. Nevertheless, the potential involvement of TTX-sensitive neuronal Na^+ channel isoforms in Purkinje AP cannot be ruled out.

It is noteworthy that $Na_V1.4$ showed a distinct pattern of staining compared to $Na_V1.5$. $Na_V1.4$ was uniformly localized to the sarcolemma, whereas $Na_V1.5$ was localized to the intercalated disks as previously shown by Maier et al. [31,32]. Interestingly, the neuronal Na^+ channel isoform such as $Na_V1.1$ is predominantly localized at the intercalated disks and around the nucleus in Purkinje myocytes [11]. These distinct localizations suggest these Na^+ channel isoforms may play different roles in Purkinje fibers. The exact functional significance of the unique sarcolemmal distribution of the skeletal muscle $Na_V1.4$ in Purkinje myocytes is not known and warrants additional studies.

In conclusion, in addition to the neuronal and cardiac Na^+ channel isoforms, we demonstrated that the skeletal muscle $Na_V1.4$ Na^+ channel isoform is present in Purkinje myocytes. Multiple Na^+ channel isoforms already identified and differently expressed in cardiac tissues may be involved in different functions in different cardiac cells. This novel finding will open the field for exploring the physiological function of the skeletal muscle $Na_V1.4$ in the cardiac Purkinje myocytes.

Acknowledgments

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