RAPID REPORT

Molecular basis of the T- and L-type Ca^{2+} currents in canine Purkinje fibres

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This study examines the molecular basis for the T-type and L-type Ca^{2+} currents in canine Purkinje cells. The I_{CaT} in Purkinje cells was completely suppressed by 200 nM kurtoxin, a specific blocker of both $Ca_v3.1$ and $Ca_v3.2$ channels. Since only $Ca_v3.2$ mRNA is expressed at high levels in Purkinje fibres, being approximately 100-fold more abundant than either $Ca_v3.1$ or $Ca_v3.3$ mRNAs, it is concluded that the $Ca_v3.2$ gene encodes the bulk of the T-type Ca^{2+} channels in canine Purkinje cells. This conclusion is consistent with the sensitivity of the current to blockade by Ni^{2+} ions ($K_D = 32\ \mu M$). For L-type channels, $Ca_v1.2$ mRNA was most abundant in Purkinje fibres but a significant level of $Ca_v1.3$ mRNA expression was also found. A comparison of the sensitivity to blockade by isradipine of the L-type currents in Purkinje cells and ventricular epicardial myocytes, which only express $Ca_v1.2$, suggests that the $Ca_v1.3$ channels make, at most, a minor contribution to the L-type current in canine Purkinje cells.

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Two types of calcium currents have been described in canine Purkinje cells: L-type (I_{CaL}) and T-type (I_{CaT}) (Hirano *et al.* 1989; Tseng & Boyden, 1989). The L-type Ca^{2+} current is ubiquitous in cardiac myocytes. In contrast, the T-type calcium current is expressed in a more restricted pattern in the heart, being found mainly in Purkinje cells, sinus node cells and embryonic myocytes (reviewed in Vassort *et al.* 2006), although it has been described at low levels in adult ventricular myocytes in some species (Mitra & Morad, 1986; Tseng & Boyden, 1989; Wang & Cohen, 2003). This expression pattern, together with the biophysical properties of the T-type Ca^{2+} channels, is consistent with a role for these channels in conduction and pacemaking (Vassort *et al.* 2006).

Three different subfamilies of genes encoding Ca^{2+} channel principal subunits have been described (Catterall *et al.* 2005). Members of the first subfamily (Ca_v1 genes) encode channels conducting typical L-type currents. In cardiac myocytes, the bulk of I_{CaL} is conducted by channels encoded by the $Ca_v1.2$ gene (CACNA1C or α1C) (Striessnig, 1999). The $Ca_v1.3$ (CACNA1D or α1D) channel may contribute to the L-type currents in atrioventricular and sinus node cells (Bohn *et al.* 2000; Platzer *et al.* 2000; Mangoni *et al.* 2003). The other two members of the Ca_v1 family are not significantly expressed in the myocardium (Striessnig, 1999; Catterall *et al.* 2005).

The second subfamily of Ca^{2+} channel principal subunit genes (Ca_v2) encodes channels conducting currents whose biophysical properties are intermediate between L- and T-type currents. These genes are the molecular correlates of the N-, P/Q- and R- types of Ca^{2+} currents (Catterall *et al.* 2005). Although some of these genes have been reported to be expressed in the heart (reviewed in Catterall *et al.* 2005), no corresponding currents have been described in normal cardiac myocytes to date.

Three genes are found in the third subfamily: $Ca_v3.1$ (CACNA1G or α1G), $Ca_v3.2$ (CACNA1H or α1H) and $Ca_v3.3$ (CACNA1I or α1I) (Perez-Reyes *et al.* 1998; Cribbs *et al.* 1998; Lee *et al.* 1999*a*; Catterall *et al.* 2005). The biophysical properties of the encoded channels are those of typical T-type Ca^{2+} currents. Ca_v3 genes have been reported to be expressed in sinus node cells (Bohn *et al.* 2000), embryonic hearts (Niwa *et al.* 2004) and Purkinje cells (Han *et al.* 2002).

Recent studies have investigated ion channel gene expression in Purkinje fibres (Shi *et al.* 1999; Han *et al.* 2002; Pourrier *et al.* 2003), but a detailed analysis of the molecular basis of Ca^{2+} currents in these cells has not been performed. In this paper, we examine the molecular basis of the canine Purkinje fibre T- and L-type calcium currents.

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Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee of Stony Brook University and Columbia University.

Isolation of RNA from Canine Left Ventricular Wall and Purkinje Fibres

Adult cross-breed dogs were killed with pentobarbital (80 mg kg^{-1}, I.V., via the cephalic vein). Free-running Purkinje fibres or portions of the left ventricular free wall were dissected and quick-frozen in liquid nitrogen. Total RNA was prepared using RNeasy columns (Qiagen). A DNase I treatment step was included to eliminate genomic DNA contamination.

Real-time PCR Analysis

Quantification of mRNA for the calcium channel genes was performed using real-time PCR analysis, as previously described (Rosati *et al.* 2006). Variability between samples was assessed by measuring 18S and 28S internal control genes. Multiple primer pairs were used for gene-specific amplification. This approach reveals any potential heterogeneity in amplification efficiencies for the different primer pairs, making the comparison of the expression levels of different genes more accurate.

Primer sequences were as follows

18S. Forward(1): CCTGCGGCTTAATTTGACTC, Reverse(1): CGGACATCTAAGGGCATCAC; Forward(2): GTTCCGACCATAAACGATGC, Reverse(2): AACTAAGAACGGCCATGCAC; Forward(3): CCCGAAGCTTTACTTTGAA, Reverse(3): GCATCGTTTATGGTCGGAAC.

28S. Forward(1): TGGGTTTTAAGCAGGAGGTG, Reverse(1): TCCTCAGCCAAGCACATACA; Forward(2): AAAGGGAGTCGGGTTCAGAT, Reverse(2): AGAGGCTGTTCACCTTGGAG; Forward(3): TCCGAAGTTTCCCTCAGGAT, Reverse(3): CCTTTTCTGGGGTCTGATGA.

Cav1.1. Forward(1): ACCGGAACAACAACTTCCAG, Reverse(1): ATGGCCTTGAACTCATCCAG; Forward(2): TCATTCTGCTGGGATCCTTC, Reverse(2): CCTCCAGTGGCGGATGAACT.

Cav1.2. Forward(1): CGATTTTTGCCAATTGTGTG, Reverse(1): CTCAGTGCCTTCACATCGAA; Forward(2): ATGAAGGCATGGATGAGGAG, Reverse(2): CCAGTGGGGCTGATTGTAGT.

Cav1.3. Forward(1): GAACAAGGACTGGTGGGAAA, Reverse(1): GAACTGAAGGCCTTTGGACA; Forward(2): TATGATGGCGCTCTTCACGG, Reverse(2): GTAGGGGTTTTTGGGGATGT.

Cav1.4. Forward(1): CCAAGAACCCACATCAATAT, Reverse(1): TTCAGTGACGGCAATATCCA.

Cav3.1. Forward(1): CAGGCAGCAATAAGGACTGA, Reverse(1): GGAGGTCTCCTGAAATCCAG; Forward(2): CATCTCTTTGGCTGCAAATT, Reverse(2): ACTGTCCACTCGCATCTTCC; Forward(3): CATCAGCATGTTGGTCATCC, Reverse(3): GAAGCTGACGTTCTGCAGGT.

Cav3.2. Forward(1): GTCAGCCACATCACCAGCTC, Reverse(1): GGGGGGCTCATCTTCTTCTT; Forward(2): GCTGCAAGTTCAGTCTGACG, Reverse(2): CTCCAAGTGGGTGGATGTCT; Forward(3): TCAACGTCATCACCATGTCC, Reverse(3): CTTGAGCAGCTTCAGAACTC.

Cav3.3. Forward(1): TCTTCAAGGACCGATGGAAC, Reverse(1): CAGACCAGCTTCCCAAAGAG; Forward(2): CATCTTTGGCTGCAAATTCA, Reverse(2): TTGGATGAGCTCTGGTCCTC; Forward(3): GCCAAGGACGTCTTTACCAA, Reverse(3): AGGAAGATGCGTTCAGTGCT.

For each gene, results obtained from the different sets of primer pairs were very consistent. All of the primer pairs were first tested using canine brain mRNA as a positive control. The specificity of the amplified products was determined by gel electrophoresis and sequence analysis.

All of the primers were targeted to amplify regions devoid of alternative splicing. When no information on splice variants for a given gene was available in the canine, we used information available for the corresponding human gene. In all of the reported experiments only a single amplification product was detected.

Electrophysiological recordings

Dissociation and recording techniques have been previously described (Tseng & Boyden, 1989). Patch pipettes were filled with an internal solution that had the following composition (mM): CsOH 125, aspartic acid 125, tetraethylammonium chloride 20, Hepes 10, Mg-ATP 5, EGTA 10 and phosphocreatine 3.6 (pH 7.3 with CsOH). A period of 5–10 min was then allowed for intracellular dialysis to begin before switching to a nominal sodium-free extracellular recording solution (mM): $CaCl_2$ 5.0, $MgCl_2$ 0.5, tetraethylammonium chloride 140, Hepes 12, dextrose 10, and 4-aminopyridine 2 (pH 7.3 with CsOH). After the appropriate controls, all recordings were performed

in the presence of 20 μM tetrodotoxin (TTX) to avoid contamination of the currents with the TTX-sensitive Ca^{2+} current (I_{CaTTX}) (Lemaire *et al.* 1995; Cole *et al.* 1997).

To separate the T- and L-type Ca^{2+} currents, different holding voltages were used. Typically, a holding voltage (V_h) of −40 mV (for the L-type current) and −70 or −90 mV (for the T-type current) was used. In preliminary experiments, we found negligible inactivation of the T-current at the holding voltages specified above. The T-type current was isolated from the L-type current by subtracting the current recorded at a V_h of −40 mV from that recorded at the more negative V_h values. TTX (Alomone Laboratories, Jerusalem, Israel or Sigma, St Louis, MO, USA), ω-conotoxin MVIIC (Alomone Laboratories), isradipine (Sigma) $NiCL_2$ (Fisher Scientific) and kurtoxin (Sigma) were added fresh on the day of the experiment.

To calculate the K_D for nickel ion blockade of I_{CaT}, data points were fitted with the Hill equation: fractional inhibition $= 1/(1 + (K_D/[Ni^{2+}]))$.

Results

Calcium currents in canine Purkinje cells

Two primary components can be observed in recordings of total calcium currents from isolated canine Purkinje cells (Fig. 1). These two components correspond to the T-type and L-type calcium currents and have been previously described (Tseng & Boyden, 1989; Hirano *et al.* 1989). The currents can be distinguished on the basis of different thresholds for activation, steady-state inactivation curves and time constants of inactivation.

In Purkinje cells, the threshold for activation of the T-type current is relatively positive compared to the T-type currents found in many other cells, being approximately −45 mV. Average values for the current density for the T-type and L-type currents in Purkinje cells are 1.8 ± 0.3 pA pF^{-1} (mean ± S.E.M., $n = 7$) and 5.5 ± 0.6 pA pF^{-1} ($n = 10$), respectively.

Toxin sensitivity of the T-type calcium current

The T-type current found in Purkinje cells could, in principle, be encoded by members of either the Ca_v3 or Ca_v2 family of calcium channel genes, because the activation threshold of the current is located at a relatively hyperpolarized membrane potential. To address this issue, we examined the sensitivity of the T-type current to two toxins that are specific blockers of channels encoded by either the Ca_v2 gene family or the Ca_v3 gene family.

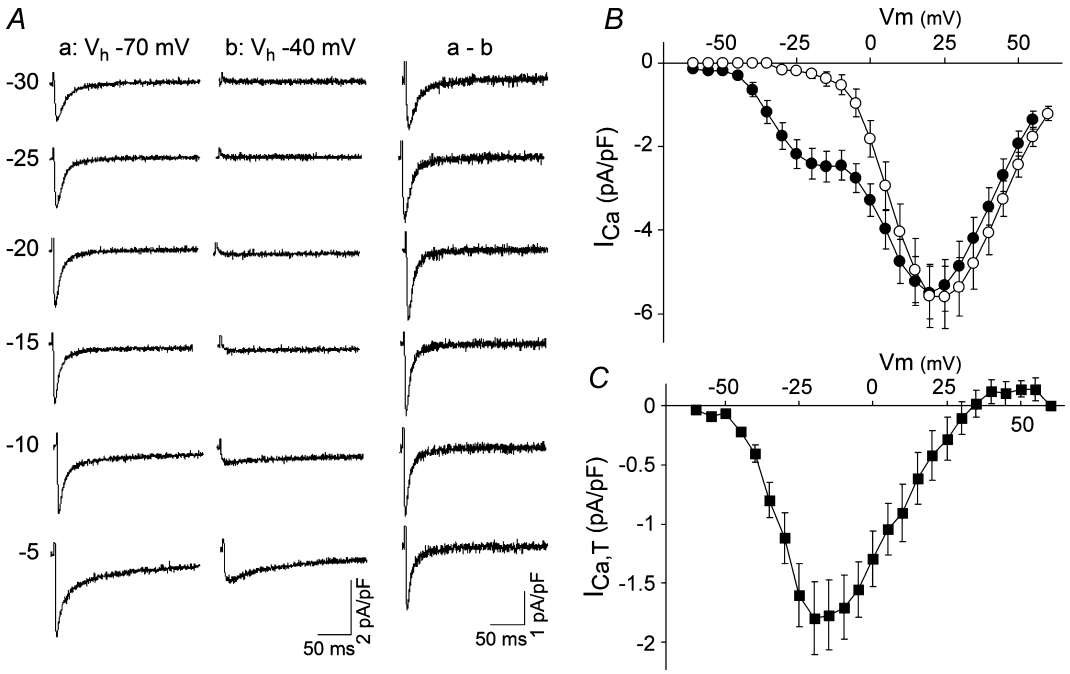

Figure 1. L- and T-type calcium currents in canine Purkinje cells
A, recordings of total calcium currents elicited in an isolated Purkinje cell in response to depolarizing voltage steps (values on the left of the corresponding traces), from holding potentials (V_h) of −70 (left column) or −40 mV (centre column), as indicated. Difference currents for each test pulse are shown in the right column (*a* – *b*). *B*, current–voltage relationship for the total calcium current elicited from V_h of either −70 mV (●, $n = 10$ cells, mean ± S.E.M.) or −40 mV (○, $n = 7$ cells, mean ± S.E.M.). *C*, current–voltage relationship of the T-type current, as isolated by subtraction of the total current at $V_h = -40$ mV from the current recorded at $V_h = -70$ mV. Mean data ± S.E.M. ($n = 7$) are shown.

ω-Conotoxin MVIIC (ω-CTX) is a specific blocker of the $Ca_v2.1$ (Sather *et al.* 1993) and $Ca_v2.2$ (Stocker *et al.* 1997) channels, as well as of the native N-type and P/Q-type Ca^{2+} currents (McDonough *et al.* 1996). Application of 1 μM ω-CTX did not result in a reduction of the peak T-type calcium current (not shown).

Kurtoxin is a potent and specific blocker of $Ca_v3.1$ and $Ca_v3.2$ channels that does not affect $Ca_v2.1$, $Ca_v2.2$ or $Ca_v2.3$ currents (Chuang *et al.* 1998). Application of this toxin results in an almost complete elimination of the low threshold component of the total calcium current (Fig. 2*A*–*C*), ruling out a major involvement of any of the Ca_v2 channel genes in I_{CaT} and indicating that the Ca_v3 genes underlie the T-type calcium current in canine Purkinje cells.

Quantitative comparison of Ca_V3 subfamily mRNA expression in Purkinje fibres

Based on the effects of kurtoxin and ω-CTX, it seemed likely that the T-type current in Purkinje cells was encoded by one or possibly both of the $Ca_v3.1$ and $Ca_v3.2$ genes. Therefore the relative mRNA expression of the Ca_v3 genes in Purkinje fibres was examined using real-time PCR.

As seen in Fig. 3*A*, only the $Ca_v3.2$ gene is expressed at significant levels in Purkinje fibres, being 100-fold more abundant than the $Ca_v3.1$ and $Ca_v3.3$ genes. This result, in combination with kurtoxin sensitivity, strongly suggests that the $Ca_v3.2$ channel makes the primary contribution to the T-type current in Purkinje cells.

It has been shown that the Ni^{2+} sensitivity of $Ca_v3.2$ channels is about 20-fold higher than for $Ca_v3.1$ channels (Lee *et al.* 1999*b*). Dose–response analysis for Ni^{2+} blockade of I_{CaT} in canine Purkinje cells yielded a K_D value for channel blockade of 32 ± 3 μM (Fig. 3*C*), indicating that this current is highly sensitive to Ni^{2+} ions (Fig. 3*B*) and confirming the primary contribution of the $Ca_v3.2$ channel to the native current.

Quantitative comparison of Ca_V1 subfamily mRNA expression in canine ventricle

The L-type calcium current in Purkinje cells has the typical properties of L-type channels encoded by the Ca_v1 subfamily of calcium channel genes (Hirano *et al.* 1989; Tseng & Boyden, 1989). To determine the molecular basis of the L-type calcium current in Purkinje cells, mRNA expression from the four known Ca_v1 genes was examined. In Purkinje fibres, $Ca_v1.2$ mRNA is the most abundant Ca_v1 gene subfamily mRNA (Fig. 4*A*). There is, however, a significant level of $Ca_v1.3$ mRNA expression.

In relative terms, $Ca_v1.2$ mRNA comprises 87% of the total Ca_v1 mRNA in Purkinje fibres, with $Ca_v1.3$ mRNA making up most of the remainder (11% of total). This differs from ventricular myocytes, where $Ca_v1.2$ mRNA comprises 98% of the total Ca_v1 mRNA and $Ca_v1.3$ contributes only 1% of the total (Fig. 4*B*).

The $Ca_v1.3$ channel is less sensitive to block by dihydropyridines (DHP) than the $Ca_v1.2$ channel (Xu & Lipscombe, 2001; Koschak *et al.* 2001). In order to test whether $Ca_v1.3$ channels make a significant functional contribution to the L-type calcium current in Purkinje cells, we compared the sensitivity of Purkinje cell and ventricular myocyte L-type currents to the DHP compound isradipine. There was no significant difference in isradipine sensitivity of I_{CaL} in Purkinje cells *versus* epicardial myocytes. The percentage inhibition

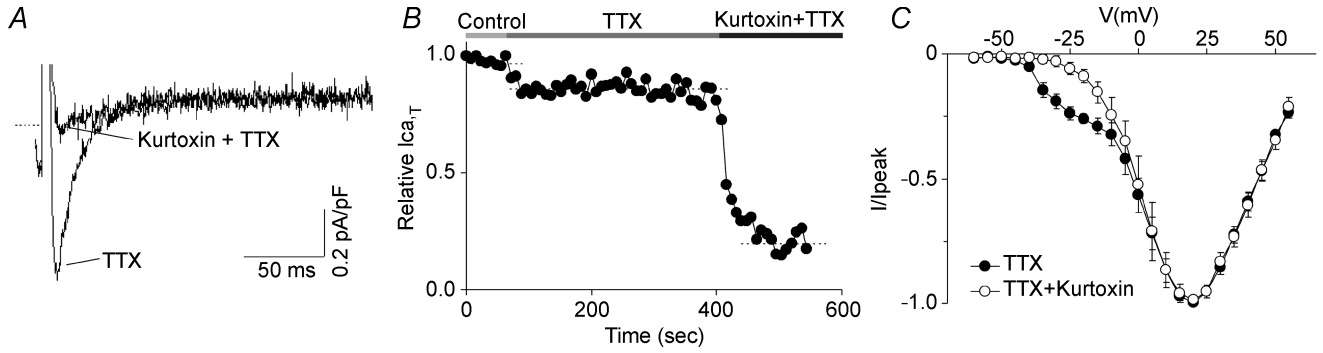

Figure 2. Effect of kurtoxin on the T-type calcium current in canine Purkinje cells
A, kurtoxin selectively blocks the T-type component of the calcium current in Purkinje cells. The current was elicited by a test pulse at −25 mV from a holding potential of −90 mV, in control conditions and after the application of 200 nM kurtoxin, as indicated. Both the control and drug recordings were performed in the presence of 20 μM TTX (see Methods for rationale). *B*, time course for the T-type calcium current inhibition by kurtoxin. The horizontal bars on top of the graph indicate the bath solution applied. *C*, average current–voltage plots obtained in $n = 3$ cells under control conditions and after application of kurtoxin (see legend). The holding potential was −70 mV and test pulses were applied from −60 to +55 mV at 5 mV intervals. Error bars represent the S.E.M.

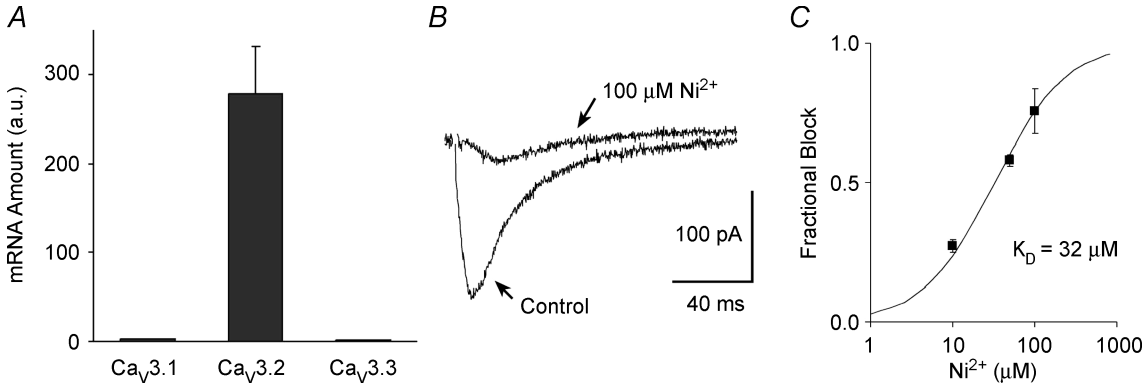

Figure 3. Molecular basis of the T-type calcium current in canine Purkinje cells
A, bar graph of relative mRNA abundance for the $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ genes in Purkinje fibres. The mRNA quantities were determined by real-time PCR and are represented in arbitrary units. Data are means ± S.E.M. ($n = 4$, each sample from 3 to 6 different hearts). *B*, sample recording of I_{CaT} in a canine Purkinje cell, in control conditions (see Methods) and in the presence of 100 μM Ni^{2+}, as indicated by the arrows. The current was elicited by a −30 mV test pulse from a holding potential of −90 mV. *C*, dose–response curve for Ni^{2+} blockade of I_{CaT}. The fractional block of the I_{CaT} is shown for three different Ni^{2+} concentrations: 10, 50 and 100 μM. Data points are average values obtained in $n = 3$ cells from 2 different hearts. Data points were fitted with the Hill equation (see Methods), yielding a K_D of 32 ± 3 μM (mean ± S.E.M.).

of I_{CaL} by 300 nM isradipine was 46.7 ± 9.9 ($n = 6$) in Purkinje cells and 40.4 ± 5.7 ($n = 5$) in epicardial myocytes (mean ± S.E.M.).

Discussion

The presence of two types of Ca^{2+} currents, L-type and T-type, in canine Purkinje fibres has been reported previously (Hirano *et al.* 1989; Tseng & Boyden, 1989). In keeping with these prior observations, in all cells tested a large I_{CaT} was detected in addition to I_{CaL}.

Three genes are known to encode I_{CaT} α subunits, namely $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$. Other channels, though, have been shown to produce T-type currents under certain conditions. In particular, $Ca_v2.1$ (α1B), $Ca_v2.3$ (α1E) and $Ca_v1.2$ (α1C) can produce T-type-like channels in the absence of auxiliary subunits (Meir & Dolphin, 1998). Moreover, $Ca_v1.3$ (α1D) has been shown to produce currents endowed with a more negative activation threshold and more rapid kinetics when compared to classic L-type currents (Lipscombe *et al.* 2004). Since subtle changes in the biophysical properties of the currents could be due to uncharacterized differences in accessory subunit composition or splice variants, we used pharmacological tools to narrow down the set of genes that could potentially encode the Purkinje cell I_{CaT} α subunit. The sensitivity of Purkinje cell I_{CaT} to kurtoxin, combined with the failure of ω-conotoxin MVIIC to block the current, indicates that the α subunit belongs to the Ca_v3 family.

The three Ca_v3 channels can be distinguished on the basis of their sensitivity to blockade by Ni^{2+} ions.

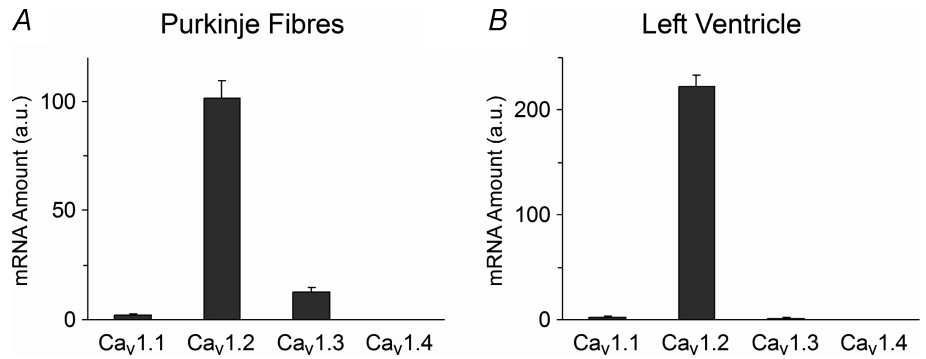

Figure 4. Ca_v1 family gene expression in the canine Purkinje fibres and left ventricle
Bar graph of relative mRNA abundance for the $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ genes in Purkinje fibres (*A*) and left ventricle (*B*). The mRNA quantities were determined by real-time PCR and are represented in arbitrary units. Data are means ± S.E.M. ($n = 3$–5, each Purkinje sample from 3 to 6 hearts; each ventricular sample from 1 heart).

Lee *et al.* (1999*b*) have shown that recombinant $Ca_v3.2$ currents are blocked by relatively low Ni^{2+} concentrations ($IC_{50} = 13\ \mu M$), while $Ca_v3.3$ and $Ca_v3.1$ currents are much less sensitive to blockade with this cation, with IC_{50} values nearly 20-fold higher ($IC_{50} = 216$ and $250\ \mu M$, respectively). We and others have previously shown that I_{CaT} in canine Purkinje cells is highly sensitive to Ni^{2+} ions (Hirano *et al.* 1989; Tseng & Boyden, 1989). In this report, we determined the K_D for Ni^{2+} blockade of I_{CaT} to be $32\ \mu M$, which is close to the IC_{50} for the block of the recombinant $Ca_v3.2$ channels, given the difficulty in recording the native current completely uncontaminated by L-type current. This result is consistent with the observation that $Ca_v3.2$ mRNA is two orders of magnitude more abundant than either $Ca_v3.1$ or $Ca_v3.3$ mRNA transcripts in Purkinje fibres and supports the hypothesis that $Ca_v3.2$ channels comprise the bulk of I_{CaT} in canine Purkinje fibres.

At this time, reliable antibodies against the Ca_v3 ion channel proteins are unavailable and expression analysis for these channels was therefore limited to mRNA. Nonetheless, the very large difference in gene expression among the Ca_v3 genes (nearly two orders of magnitude) suggests that a qualitative discordance between mRNA and protein expression for the Ca_v3 channels is unlikely.

Our Ca_v3 gene expression data in Purkinje fibres (Fig. 3*A*) are at variance with an earlier report (Han *et al.* 2002). There are several potential problems that can arise with the application of PCR for mRNA quantification (Nolan *et al.* 2006) and it is likely that the discordance of the results resides in technical differences between the two studies. Han *et al.* (2002) used competitive PCR, whereas real-time PCR was used in this report. More importantly, multiple-primer pairs were used in the current study to detect potential variation in amplification efficiency for different primer pairs, whereas only a single primer pair was used by Han *et al.* (2002). In addition, all reaction products from the experiments described in this report were sequenced to confirm that the correct amplicon was obtained. While we do not have a definitive explanation for this discrepancy, the molecular data reported here are entirely consistent with the pharmacology of the I_{CaT} in canine Purkinje cells.

In ventricular myocytes, I_{CaL} is encoded by the $Ca_v1.2$ gene. This gene is also the most abundantly expressed member of the Ca_v1 subfamily in canine Purkinje fibres (Fig. 4*A*). $Ca_v1.3$ mRNA is also expressed at significant levels in canine Purkinje fibres, suggesting a possible contribution of this channel to I_{CaL} in Purkinje cells. To test this possibility, we compared the sensitivity of the L-type current in Purkinje cells and ventricular myocytes to isradipine. Since the $Ca_v1.2$ channel is more sensitive to dihydropyridine blockade than the $Ca_v1.3$ channel (Xu & Lipscombe, 2001; Koschak *et al.* 2001; Lipscombe *et al.* 2004), a significant contribution of the latter gene to the I_{CaL} would be expected to result in a lower sensitivity of the current to isradipine. The results indicate that there is no significant difference in the isradipine sensitivity of I_{CaL} in the two tissues. Although this assay has limited sensitivity, it is reasonable to conclude that the $Ca_v1.3$ channel does not contribute to the I_{CaL} in canine Purkinje cells out of proportion to the relative abundance of its cognate mRNA.

Taken together, the pharmacology and gene expression data support the conclusion that the primary component of the T-type calcium current in canine Purkinje cells is encoded by the $Ca_v3.2$ gene and the Purkinje cell L-type current is encoded primarily by the $Ca_v1.2$ gene.

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