Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

Neuroscience xx (2006) xxx

TEMPERATURE DEPENDENCE OF T-TYPE CALCIUM CHANNEL GATING

M. IFTINCA,[a,b,c] B. E. McKAY,[b,c] T. P. SNUTCH,[d] J. E. McRORY,[a,c] R. W. TURNER[b,c] AND G. W. ZAMPONI[a,c]*

[a]*Department of Physiology and Biophysics, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Canada T2N 4N1*

[b]*Department of Anatomy and Cell Biology, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Canada T2N 4N1*

[c]*Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Canada T2N 4N1*

[d]*Michael Smith Laboratories, University of British Columbia, Vancouver, Canada V6T 1Z4*

Abstract—T-type calcium channel isoforms are expressed in a multitude of tissues and have a key role in a variety of physiological processes. To fully appreciate the physiological role of distinct channel isoforms it is essential to determine their kinetic properties under physiologically relevant conditions. We therefore characterized the gating behavior of expressed rat voltage-dependent calcium channels (Ca_v) 3.1, Ca_v3.2, and Ca_v3.3, as well as human Ca_v3.3 at 21 °C and 37 °C in saline that approximates physiological conditions. Exposure to 37 °C caused significant increases in the rates of activation, inactivation, and recovery from inactivation, increased the current amplitudes, and induced a hyperpolarizing shift of half-activation for Ca_v3.1 and Ca_v3.2. At 37 °C the half-inactivation showed a hyperpolarizing shift for Ca_v3.1 and Ca_v3.2 and human Ca_v3.3, but not rat Ca_v3.3. The observed changes in the kinetics were significant but not identical for the three isoforms, showing that the ability of T-type channels to conduct calcium varies with both channel isoform and temperature.

Key words: temperature, physiological conditions, calcium channel, T-type, kinetics.

Low-threshold or T-type calcium channels are expressed in a variety of tissues including nervous tissue, heart, smooth muscle, kidney, sperm, and various endocrine glands (Berridge et al., 2000; Huguenard, 1996; Perez-Reyes, 2003). These channels are thought to play an important role in controlling intracellular calcium levels, to regulate cellular processes such as neuronal excitability, synaptic plasticity, smooth and cardiac muscle contraction, reproduction and to contribute to secretion of hormones and neurotransmitters (Berridge et al., 2000; Huguenard, 1996; Perez-Reyes, 2003).

The mammalian genome encodes genes for three distinct subtypes of T-type voltage-dependent calcium channel (Ca_v3.1, Ca_v3.2, and Ca_v3.3). Each of the three subtypes is subject to alternative splicing, resulting in a variety of different isoforms that each have distinct biophysical, modulatory, and pharmacological properties (McRory et al., 2001; Perez-Reyes, 2003). While the presence of T-type channels has been demonstrated in a variety of central neurons (Huguenard, 1996; Perez-Reyes, 2003) their precise roles in regulating neuronal firing have not been fully determined since there are still no specific and selective modulatory drugs (Cavelier and Bossu, 2003; Raman and Bean, 1999; Swensen and Bean, 2003). Transient expression systems have been used extensively to determine the biophysical characteristics of T-type channel isoforms in isolation. However, these studies are typically carried out at room temperature. Correlating the properties of expressed T-type channels at room temperature with their role in regulating the excitability of neurons at physiological temperatures has been challenging. In fact, temperature is known to accelerate gating kinetics and shift the position of the current–voltage relation of native low threshold channels in thalamic neurons (Coulter et al., 1989), dorsal root ganglion (DRG) neurons (Nobile et al., 1990), neuroblastoma cells (Narahashi et al., 1987), and GH_3 cells (Rosen, 1996). However, these cells express a multiplicity of calcium channels that potentially include several T-type isoforms as well as high threshold calcium channels. Given the difficulty of cleanly separating native low and high threshold calcium currents due to overlapping open probabilities at some voltages, and because selective blockers of specific T-type channels are unavailable, it remains unknown to what extent temperature can regulate the properties of individual T-type calcium channel isoforms.

To answer this question we have examined the effects of temperature on the gating behavior of individual T-type calcium channel subtypes transiently expressed in tsA-201 cells. Because we wanted to study these channels in a physiological setting we also used calcium at a physiological concentration of 1.5 mM as the charge carrier and the properties of each T-type channel isoform were measured at room and physiological temperatures. Our data show that the biophysical characteristics of the channels are dramatically altered at 37 °C compared with those observed at room temperature. The gating kinetics, including rates of activation, inactivation, and recovery from inacti-

*Correspondence to: G. W. Zamponi, Department of Physiology and Biophysics, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Canada T2N 4N1. Tel: +1-403-220-8687; fax: +1-403-210-8106.
E-mail address: zamponi@ucalgary.ca (G. W. Zamponi).

Abbreviations: ANOVA, analysis of variance; Ca_v, voltage-dependent calcium channel; Ca_v3.3*h*, human Ca_v3.3; cDNA, complimentary DNA; eGFP, enhanced green fluorescent protein; EGTA, ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; GTP, guanosine 5-triphosphate; PCR, polymerase chain reaction; TEA, tetraethylammonium chloride; V_a, half activation potential; V_h, half inactivation potential.

0306-4522/06$30.00+0.00 © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2006.07.010

vation were all accelerated, whereas the voltage-dependences of activation and inactivation were affected to a lesser extent. Our results reveal dramatic shifts in the gating kinetics of Ca_v3 isoforms at physiological temperatures that will be important to consider when assessing the potential role of these channels in shaping cell activities.

EXPERIMENTAL PROCEDURES

Calcium channel complimentary DNAs (cDNAs)

Rat $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ cDNAs were the same as those isolated and described by McRory et al. (2001). Our rat $Ca_v3.1$ sequence (AF29012) is identical to that isolated by Perez-Reyes and colleagues (NM_031601; Perez-Reyes et al., 1998). The rat $Ca_v3.3$ construct used in our study constitutes the "b" C-terminal splice isoform. We noted that the GenBank entry for this sequence, originally published by McRory et al. (2001) displayed a number of sequence differences to other published sequences, including several amino acid changes as well as an apparent deletion of three amino acids in the IIIS4 region. Because a recent study examining splice variants of $Ca_v3.3$ could not verify the existence of a variant lacking these residues (Murbartian et al., 2002), we re-sequenced the $Ca_v3.3$ cDNA and determined that our cDNA did in fact include the three residues. In addition, the majority of the sequence differences relative to other published sequences also proved to be sequence errors. There are four remaining differences to the Perez-Reyes, 2003 clone within the four major transmembrane domains, however, our amino acid sequence in these four regions matches that of the rat genome and two of them are also found in expressed sequence tags. The corrected sequence has now been posted on GenBank (accession number AF290214). We also resequenced the entire rat $Ca_v3.2$ cDNA and changed the GenBank entry for the rat $Ca_v3.2$ clone (accession number AF290213.2). We note that all three cDNAs used in our study were isolated from cDNA libraries and never subjected to polymerase chain reaction (PCR) amplification, hence ruling out PCR errors. Any remaining amino acid differences relative to other posted sequences are then perhaps due to allelic differences or alternate splicing. The human $Ca_v3.3$ cDNA (denoted as $Ca_v3.3h$) was as described previously (Monteil et al., 2000).

The Cav 3.3*h* clone is the "a" splice variant (accession number NP_066919) and has several differences in comparison to our rat Cav3.3 clone (accession number AF290214). Since the two clones are from different species, there are a number of individual nucleotide differences that result in single amino acid changes throughout the sequence. In addition, there are several sites in both clones that add or remove nucleotides while maintaining the reading frame. The largest splice difference is within the I–II linker and results in the rat clone having 108 nucleotides (36 amino acids) removed when compared with the human clone. The other area which differs substantially is at the beginning of the carboxyl tail where intron–exon boundaries differ between the rat and human cDNAs and thus result in a region of 50 amino acids that possess low identity.

Cell culture and transient transfection

Tissue culture and transfection of human embryonic kidney tsA-201 cells were performed as previously described (Beedle et al., 2002). Briefly, tsA-201 cells were grown to 85% confluence at 37 °C (5% CO_2) in Dulbecco's modified Eagle's medium (+10% fetal bovine serum, 200 units/ml penicillin and 0.2 mg/ml streptomycin; Invitrogen, Carlsbad, CA, USA). Cells were dissociated with trypsin (0.25%)-EDTA before plating on glass coverslips. Wild type $Ca_v3.1$, 3.2 and 3.3 α_1 subunits (8 μg) and enhanced green fluorescent protein (eGFP) marker DNA (1.5 μg) were transfected into cells by the calcium phosphate method. Cells were transferred to 28 °C 24 h after transfection, and electrophysiological recordings were conducted 2 days later.

Electrophysiological measurements

For recordings cells were placed into a 2 ml bath containing a solution of 1.5 mM Ca^{2+} (in mM: 128.25 CsCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 10 Hepes, 25 D-glucose, pH 7.2 adjusted with CsOH) on the stage of an epi-fluorescence microscope (Diaphot-TMD, Nikon Inc., Melville, NY, USA). In a subset of experiments, CsCl was replaced by NaCl (128.5 mM). In another subset of experiments, CsCl was substituted with tetraethylammonium chloride (TEA)–Cl (in mM: 130 TEA–Cl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 10 Hepes, 25 D-glucose, pH 7.2 adjusted with TEA–OH). Finally, some experiments were conducted in a solution containing 160 mM TEA–Cl (in mM: 160 TEA–Cl, 2 $CaCl_2$, 10 Hepes, pH 7.2 adjusted with TEA–OH). Data obtained with different types of external recording solutions were not combined, and are presented for comparative purposes in Fig. 5.

tsA-201 cells expressing the transfected Ca_v3 isoforms were identified via eGFP fluorescence. Cells were voltage clamped and membrane currents were measured using conventional whole-cell patch clamp (Hamill et al., 1981). Borosilicate glass pipettes were pulled with a Sutter P-87 puller (Sutter Instruments Co., Novato, CA, USA) and polished to 2–4 MΩ resistance with an MF 830 microforge (Narishige Co., Tokyo, Japan). Pipettes were filled with an internal solution containing (in mM): 137.4 CsCl, 0.1 EGTA, 10 Hepes, 0.3 $MgCl_2$, pH 7.2 adjusted with CsOH. In some cases, the internal solution was supplemented with 2 mM ATP and 0.6 mM guanosine 5-triphosphate (GTP). Data obtained with different types of internal solutions were not pooled. Recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). All solutions were prepared at room temperature and heating from room temperature to 37 °C induced a pH reduction of ~0.1 for both internal and external solutions.

The majority of the recordings were done using separate cells in 21 °C or 37 °C. Only a limited number of cells were used in experiments in which the temperature was increased from 21 °C to 37 °C, or alternatively where temperature was decreased from 37 °C to 21 °C. The results obtained with these two paradigms were statistically indistinguishable from each other, and from our data with single temperatures per cell. To control bath temperature we used a Delta T4 Culture Dish Controller (Bioptech, Butler, PA, USA) which required less than one minute to raise the temperature from ambient to physiological. The polystyrene ring of this device was bonded to the bottom surface of the recording chamber. The variation of temperature from the set value was ±0.5 °C. Voltage clamp protocols were applied using pClamp 9.0 software (Axon Instruments, Foster City, CA, USA). Data were filtered at 1 kHz (eight-pole Bessel) and digitized at 10 kHz with a Digidata A/D converter (Axon Instruments). The filter dead time in these experiments was 0.179 ms, and thus eightfold lower than the fastest deactivation time constant measured, allowing for adequate resolution of tail current kinetics. Our ability to properly clamp the faster calcium currents at physiological temperatures was confirmed by using a tail current envelope protocol as described by Matteson and Armstrong (1984); this was done in a separate set of experiments conducted under experimental conditions that reflected those used for the data included in the manuscript (n=6 at each temperature, data not shown). Average cell capacitance was of 23.3±0.9 pF. Typically only currents smaller than 1200 pA (with a current density of 39.68±5.57 pA/pF, 28.05±4.26 pA/pF, 37.21±6.02 pA/pF, and 31.24±2.03 pA/pF for 3.1, 3.2, 3.3 and 3.3*h* respectively) were used for analysis, hence the voltage errors were consistently smaller than 5 mV. Only those cells that exhibited a stable voltage control throughout the recording were used for analysis.

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

Data analysis

Data analysis and offline leak subtraction were completed in Clampfit 9.0 (Axon Instruments), and all curves were fitted using Origin 7.0 analysis software (OriginLab, Northampton, MA, USA). Steady-state inactivation curves were fitted using the Boltzmann function:

$$I=\frac{1}{1+e^{(V-Vh)/kinact}}$$

where V_h corresponds to the half inactivation potential and *kinact* is the slope factor. Current–voltage (*IV*) plots were fitted using the Boltzmann equation:

$$I=\frac{1}{1+e^{-(V-V_a)/kact}}\times G\times(V-Erev)$$

where *Erev* is the reversal potential and *G* is the maximum slope conductance and Va the half activation potential.

For the data described in Fig. 2B, activation time courses, τ_a, were obtained by mono-exponential fits to the late rising phase of the raw current traces.

$$I(t)=Ipeak(1-e^{(-t/\tau a)})$$

Inactivation and deactivation time courses were fitted using either a mono-exponential function

$$I(t)=Ipeak(e^{(-t/\tau)})$$

or in the case of inactivation time courses obtained at 37 °C, a bi-exponential function

$$I(t)=A(e^{(-t/\tau 1)})+B(e^{(-t/\tau 2)})$$

Statistics

All averaged data are plotted as mean±S.E.M. and numbers in parentheses reflect the number of cells (*n*). Statistical analyses were completed with SigmaStat 2.0 software (Access Softek Inc., Berkeley, CA, USA), using paired *t*-test for all the results obtained before and after treatment in the same cells, and unpaired *t*-tests when data were obtained at the two temperatures from two separate sets of cells, and one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple comparisons, with the criterion for statistical significance set at $P<0.05$.

RESULTS

Effects of temperature on T-type calcium channel activation and inactivation

To determine the temperature-dependence of Ca_v3 calcium channel gating, we exogenously expressed the three different rat brain T-type calcium channel isoforms ($Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$, previously described by McRory et al. (2001)) in tsA-201 cells, and performed whole cell voltage-clamp recordings at 21 °C and 37 °C. All calcium currents were evoked by step depolarizations from a holding potential of −110 mV. At 21 °C $Ca_v3.1$ channels exhibited the fastest gating kinetics followed by $Ca_v3.2$ and $Ca_v3.3$. While the gating characteristics of rat $Ca_v3.1$ and $Ca_v3.2$ were found to be similar to those reported previously by other investigators, the rat $Ca_v3.3$b channel examined here displayed a voltage-dependence of activation that was about 20 mV more hyperpolarized when compared with data obtained with the rat $Ca_v3.3$a isoform (see Murbartian et al., 2002) or with human $Ca_v3.3$ (Monteil et al., 2000). Because of this unique gating behavior of the rat $Ca_v3.3$ construct, we conducted a series of parallel experiments using $Ca_v3.3h$ (see Table 1) and obtained results that were in close agreement with those previously described by Monteil et al. (2000), indicating that the unique properties of our $Ca_v3.3$ isoform are not due to problems in recording $Ca_v3.3$ channel activity under our experimental conditions. Instead, as we will outline below, the specific properties observed with rat $Ca_v3.3$ are intrinsic to this particular subunit and modulated strongly by the composition of extracellular recording solutions (see Discussion).

In general, increasing the temperature from 21 °C to 37 °C resulted in a large and significant reduction in the time constants of activation and inactivation for all three Ca_v3 channel subtypes as well as the $Ca_v3.3h$ isoform (not shown), but resulted in smaller changes in the steady-state relationships (Fig. 1 A and B, Fig. 2A–C). We also examined whether temperature affected peak current amplitude. To eliminate expression levels as a source of variability, current amplitudes for each isoform were measured from the same cell at both temperatures. Increasing the temperature increased the current amplitude by 1.8-fold for $Ca_v3.1$ ($n=14$, $P=0.002$), 2.1-fold for $Ca_v3.2$ ($n=11$, $P=0.001$) and 1.6-fold for $Ca_v3.3$ ($n=11$, $P=0.002$; paired *t*-test). Similar results were obtained with $Ca_v3.3h$ (1.8-fold increase, data not shown). There was no difference in the observed effects when temperatures were either ramped up from 21 °C, or down from 37 °C. Although we did not determine the temperature coefficients (Q_{10}) to facilitate comparisons between the results obtained with different isoforms we calculated the ratio (Q) of the rate constants measured at the two temperatures.

The voltage-dependence of activation underwent a small but significant (~9 mV) hyperpolarizing shift for the $Ca_v3.1$ and $Ca_v3.2$ channels upon increasing the temperature ($P<0.04$, $Q=1.15$ and 1.2, respectively), whereas that of rat $Ca_v3.3$ or $Ca_v3.3h$ was not significantly altered (Fig. 2A, Table 1). The slope of the voltage-dependence of activation did not significantly change with temperature for any of the isoforms (Table 1). Increasing the temperature also resulted in a hyperpolarizing shift in half-inactivation potential for $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3h$ and a small depolarizing shift for the rat $Ca_v3.3$ (Fig. 2A, Table 1); effects that were statistically significant only for the $Ca_v3.2$ subtype ($P=0.04$, $Q=1.15$) and $Ca_v3.3h$ ($P=0.01$, $Q=1.19$) (see Table 1). The combined effects of temperature on half activation and half inactivation potentials further resulted in a hyperpolarizing shift in the position of the window current (i.e. the overlap between activation and inactivation curves) for $Ca_v3.1$ and $Ca_v3.2$ (Fig. 2A), which would allow these channels to be active at neuronal resting membrane potentials (Perez-Reyes, 2003).

The time course of inactivation for all three isoforms was significantly accelerated at 37 °C compared with 21 °C. This shift in inactivation was apparent at all voltages tested for $Ca_v3.1$ and $Ca_v3.3$, but was observed only below −20 mV for $Ca_v3.2$ (Fig. 2C). It should be noted that the time course of inactivation was adequately described by a

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

Table 1. Effect of temperature on the half activation (V_a) and half inactivation (V_h) potentials of Ca_v3 isoforms

	$Ca_v3.1$		$Ca_v3.2$		$Ca_v3.3$		$Ca_v3.3h$	
	21°C	37°C	21°C	37°C	21°C	37°C	21°C	37°C
Activation								
V_a (mV)	−52.4±0.7 (39)	−60.5±0.9 (25)*	−42.9±0.8 (27)	−51.5±1.0 (27)*	−72.9±1.1 (18)	−73.5±1.3 (11)	−55.5±1.1 (19)	−56.8±0.9 (18)
Q		1.15		1.2		1.01		1.02
k_{activ}	5.4±0.3	4.2±1.1	6.2±0.9	5.8±0.3	4.6±1.1	4.4±0.9	5.9±0.1	5.3±0.2
Q		0.78		0.94		0.96		0.89
Inactivation								
V_h (mV)	−72.2±1.1 (17)	−75.4±4.3 (9)*	−64.2±2.2 (8)	−73.7±1.2 (5)*	−78.3±1.9 (10)	−73.4±2.5 (6)	−69.0±1.9 (8)	−82.1±1.5 (10)*
Q		1.04		1.15		0.94		1.19
$k_{inactiv}$	6.9±1.1	7.3±0.6	8.8±0.4	9.1±0.3	6.5±0.2	5.6±0.5	6.9±0.8	5.2±0.8
Q		1.06		1.03		0.86		0.75
Deactivation								
$\tau_{deactivation}$ (ms)	1.3±0.1 (6)	1.5±0.2 (6)	1.2±1.0 (6)	1.0±0.2 (4)	1.8±0.5 (5)	1.1±0.0 (4)	1.9±0.1 (13)	0.8±0.1 (12)*
Q		1.15		0.83		0.56		0.42
Recovery								
$\tau_{recovery}$ (ms)	310.1±4.3 (10)	123.6±24.9 (8)*	527.8±34.5 (8)	184.1±31.9 (6)*	615.2±86.2 (11)	205.3±3.6 (6)*	620.4±52.9 (8)	183.8±1.8 (8)*
Q		0.4		0.35		0.33		0.3

k_{activ} and $k_{inactiv}$ are the slope factors for the activation and inactivation curves. Half activation and half inactivation potentials and the slopes for the activation and inactivation curves were determined from Boltzmann fits to the data obtained from individual cells and then averaged. The time-constants of deactivation ($\tau_{deactivation}$) obtained at −120 mV are also presented. The values shown for recovery from inactivation ($\tau_{recovery}$) were measured at −100 for 3.1 and 3.2 and at −110 for both rat and human 3.3 isoforms. To facilitate comparisons at the two temperatures we determined the coefficient Q. Numbers in parentheses reflect numbers of cells. Asterisks denote statistical significance relative to room temperature (unpaired *t*-test).

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

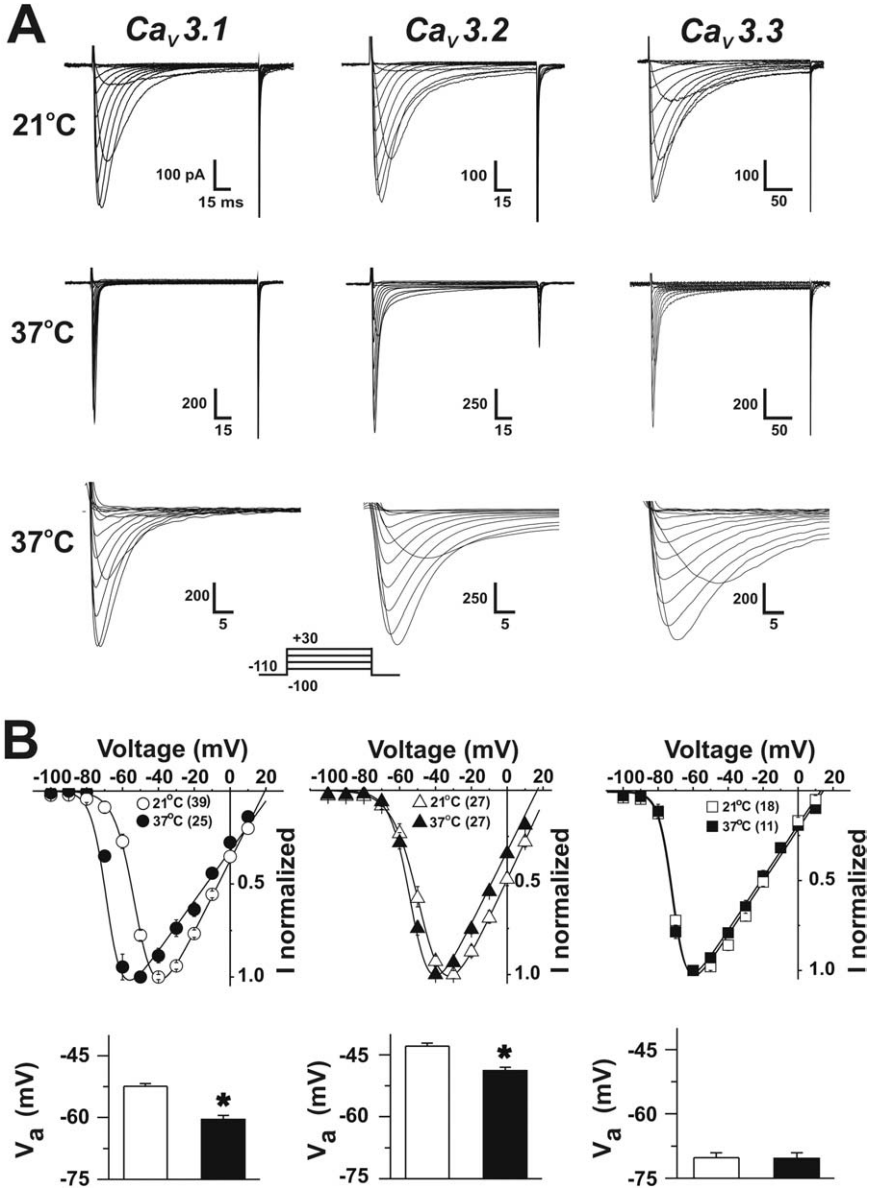

Fig. 1. Effect of temperature on Ca_v currents. (A) Representative families of whole-cell currents recorded from tsA-201 cells expressing rat $Ca_v3.1$, $Ca_v3.2$ or $Ca_v3.3$ calcium channels at 21 °C (top) and 37 °C (middle) and at 37 °C on an expanded time scale to better illustrate the time courses of activation (bottom traces). The current traces were elicited by a series of step depolarizations from a holding potential of −110 mV. The duration of the voltage steps was 200 ms for $Ca_v3.1$ and $Ca_v3.2$, and 250 ms for $Ca_v3.3$. Note the rapid activation and inactivation kinetics for all three channels at 37 °C. (B) Corresponding ensembles of whole cell current–voltage relations for each isoform at 21 °C and 37 °C. Each individual current–voltage relation was normalized and then averaged. The solid lines are Boltzmann fits to the ensemble data. The bar charts compare half-activation potentials obtained for the three Ca_v3 isoforms at both temperatures. In this case, current–voltage relations from individual experiments were fitted separately, and the mean and S.E.M. of the half-activation potentials plotted. Asterisks denote statistical significance at the 0.05 level (unpaired-*t*-test).

single exponential at 21 °C whereas a second slower decay component was apparent at 37 °C. The fast component accounted for the vast majority of the current decay, especially at potentials more depolarized than −40 mV for $Ca_v3.1$ and more depolarized than −20 mV for the other two channel types. Of particular note, the temperature dependent changes in inactivation kinetics were especially pronounced for $Ca_v3.3$ (rat and human), such that this channel no longer displayed the unusually slow inactivation kinetics present at 21 °C that has often been identified as a defining characteristic of this T-type channel isoform (Figs. 1C, 2C). Nonetheless, $Ca_v3.3$ channels still inactivated more slowly than the other T-type channel subtypes.

Effects of temperature on T-type calcium channel recovery from inactivation and deactivation

Another key feature of T-type calcium channel activity is the time required for recovery from inactivation, as this is a

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

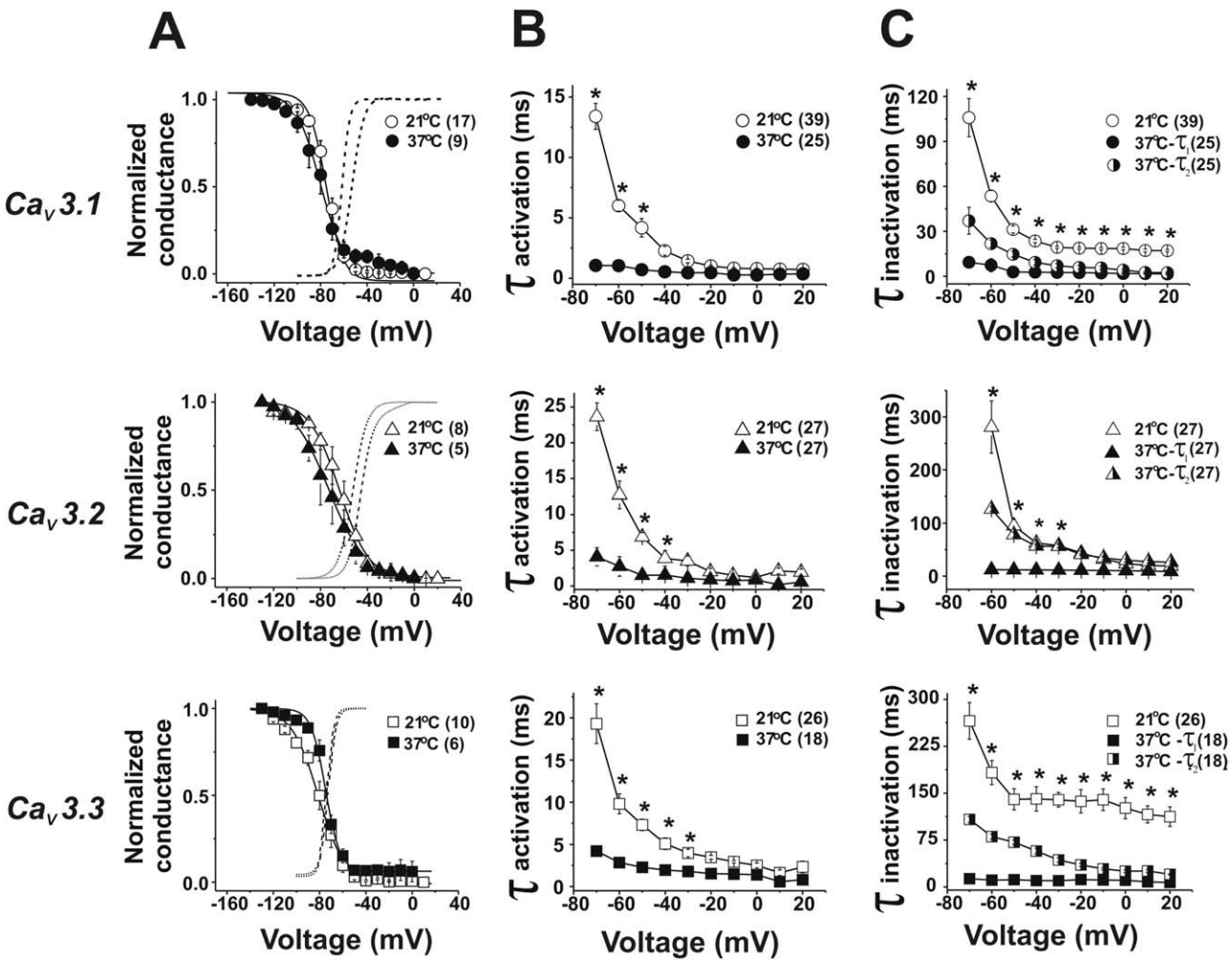

Fig. 2. Activation and inactivation kinetics are changed by temperature. (A) Ensemble steady state activation curves (dashed lines) and inactivation curves (solid lines) obtained for the three rat Ca_v3 isoforms. Cells were depolarized to various holding potentials for 2 s followed by a test pulse to −30 mV. All solid lines are Boltzmann fits to the ensemble data set. The activation curves were determined from the I–V relations shown in Fig. 1. (B) Time constants of activation of the three isoforms plotted as a function of voltage. (C) Time constants of inactivation plotted as a function of voltage. Note that the scaling of the ordinates in panels B and C differs among the three channel types. Current at 21 °C was fit with a single exponential and at 37 °C using two exponentials to fit a fast (fully shaded shapes) and slow (half-shaded shapes) inactivation time constant. Both the activation and inactivation time constants were obtained from exponential fits to raw current traces at various test potentials. Asterisks denote statistical significance of the fast inactivating component relative to the values obtained at 21 °C (unpaired *t*-test).

determinant of the refractory period after termination of an action potential or a burst. Analysis of the recovery time courses revealed a significant decrease in the time constant for recovery from inactivation at 37 °C compared with 21 °C and measured at −120 mV for $Ca_v3.1$ (n=10 and 8, respectively, P=0.02, Q=0.4), $Ca_v3.2$ (n=8 and 6, respectively, P=0.01, Q=0.35), and rat $Ca_v3.3$ (n=11 and 6, respectively, P=0.01, Q=0.33) (Fig. 3A–C, Table 1). Analogous results were obtained with the $Ca_v3.3h$ isoform (data not shown, Q=0.3). Interestingly, similar recovery times were observed for all three isoforms at 37 °C, indicating that they display a similar ability to recover from the inactivated state at physiological temperatures (but see below) (Fig. 3D). We also examined recovery from inactivation at two additional membrane potentials (−80 and −100 mV for $Ca_v3.1$ and $Ca_v3.2$; and −90 and −110 mV for $Ca_v3.3$). As shown in Fig. 3E and F, the time constants for recovery from inactivation were significantly decreased at 37 °C irrespective of membrane potential. However, unlike at more negative recovery potentials, at −80 mV $Ca_v3.2$ recovered from inactivation more slowly than $Ca_v3.1$ ($Ca_v3.1$: at 21 °C: 444.1±76.2 ms (n=5) and at 37 °C: 197.3±15.5 ms (n=5, P=0.02, Q=0.44), $Ca_v3.2$: at 21 °C: 722.9±73.0 ms (n=4) and at 37 °C: 488.7±48.7 ms (n=5, P=0.03, Q=0.68)) (note that recovery could not be examined for rat $Ca_v3.3$ at −80 mV because of its more negative activation and inactivation range).

A further kinetic feature analyzed with respect to temperature was deactivation gating (Fig. 4, Table 1). The tail currents of the three isoforms were fitted with single exponential functions to obtain deactivation time constants. The

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

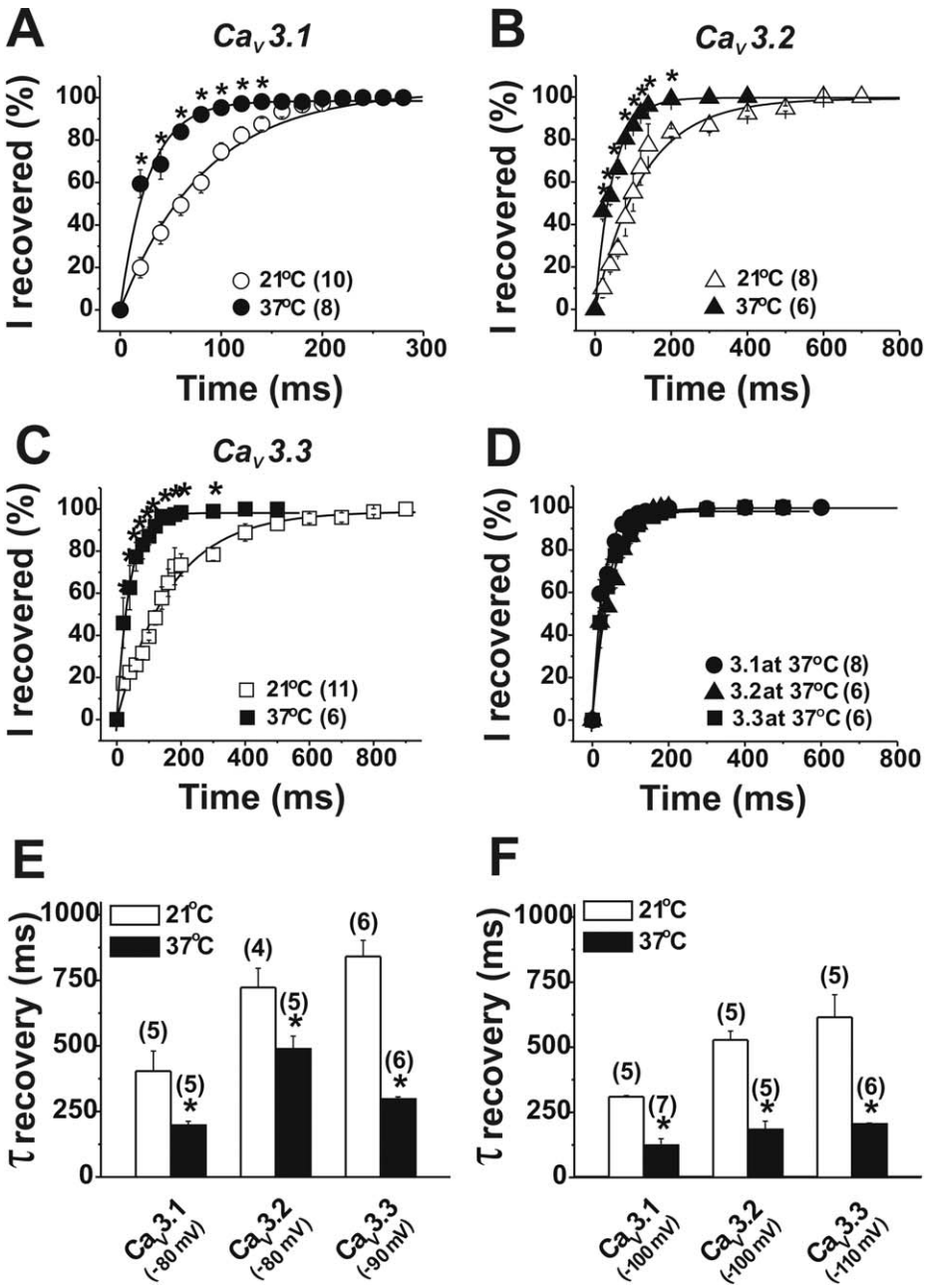

Fig. 3. Recovery from inactivation is faster under physiological temperature. Recovery from fast voltage-dependent inactivation, for rat $Ca_v3.1$ (A), $Ca_v3.2$ (B) and $Ca_v3.3$ (C) channel isoforms, was determined by application of a 2 s conditioning pulse to −30 mV, followed by a variable recovery period at a potential of −120 mV, before application of a second test pulse to −30 mV. The values plotted reflect the ratio of the current amplitudes elicited by the test and conditioning pulses, with the current elicited by the initial pulse considered to represent 100%. Solid lines are fits to the ensemble data. Numbers in parentheses reflect numbers of experiments, and asterisks denote statistical significance relative to 21 °C (unpaired *t*-test). (D) Superimposed plots of recovery from inactivation for each of the channel isoforms at 37 °C reveal similar time courses for recovery from inactivation at physiological temperature. (E, F) Comparison of the recovery kinetics for the three channels at 21 °C and 37 °C at −80 and −100 mV for $Ca_v3.1$ and 3.2; and at −90 and −110 mV for rat $Ca_v3.3$. Asterisks denote statistical significance (unpaired *t*-test). Numbers in parentheses reflect numbers of cells.

time constants were similar at both temperatures for $Ca_v3.2$ regardless of the membrane potentials tested (Fig. 4C, Table 1), whereas they became significantly different at more depolarized potentials for $Ca_v3.1$ and rat $Ca_v3.3$ (Fig. 4B, D). Interestingly, at both temperatures the rat and human $Ca_v3.3$ channel showed the slowest deactivation kinetics compared with the other two isoforms irrespective of the holding potential, suggesting that this channel subtype may have an energetically more stable open conformation. At 37 °C the deactivation time constants were comparable for the three rat isoforms at potentials more negative than −100 mV, but, rat $Ca_v3.3$ was substantially

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

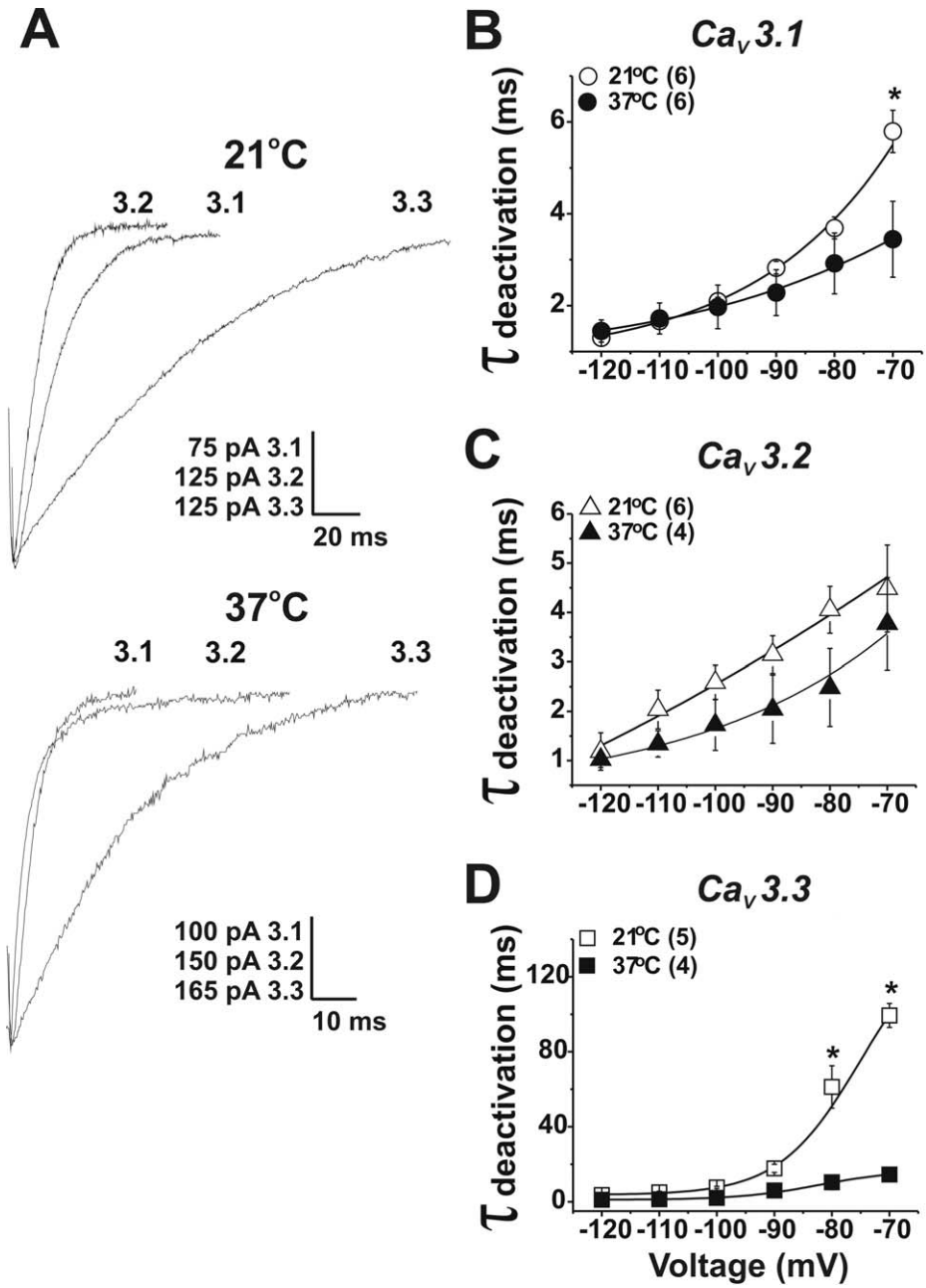

Fig. 4. Deactivation kinetics change with temperature. (A) Representative tail currents for the three rat Ca_v3 channel subtypes at 21 °C and at 37 °C. These currents were evoked by stepping from −100 mV to −30 mV, followed by a repolarization to −70 mV. These traces were chosen because they show more dramatic changes and were arbitrarily scaled to overlap at their peaks in order to facilitate comparison. Note the difference in time scale for traces measured at 21 °C and 37 °C. (B–D) Deactivation time constants obtained for $Ca_v3.1$, $Ca_v3.2$ and rat $Ca_v3.3$ plotted as a function of repolarization potential. Data were obtained by single exponential fits to the raw tail currents. Asterisks denote statistical significance relative to 21 °C (unpaired *t*-test), and numbers in parentheses the number of cells.

slower to deactivate at typical neuronal resting potentials. Moreover, the values obtained for $Ca_v3.3h$ channels were smaller than those seen with the rat $Ca_v3.3$ isoforms, but still showed temperature dependence ($Q=1.15$ for $Ca_v3.1$; $Q=0.83$ for $Ca_v3.2$; $Q=0.56$ for $Ca_v3.3$; and $Q=0.42$ for $Ca_v3.3h$) (Table 1).

Collectively, our data indicate a pronounced temperature dependence of T-type calcium channel gating that varies in magnitude with channel isoform.

DISCUSSION

It is well-known that voltage-gated ion channels display highly temperature-dependent gating characteristics (Hille, 2001). Previous work in neurons and neuron-derived cell lines revealed a temperature dependence of the biophysical properties for both low and high threshold calcium currents (Narahashi et al., 1987; Coulter et al., 1989; Nobile et al., 1990; Rosen, 1996). Because these preparations

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

express multiple calcium channel subtypes with potentially overlapping biophysical and pharmacological properties, they do not provide access to the characteristics of individual calcium channel isoforms. This is the first systematic approach to compare the functional characteristics of individual, transiently expressed Ca_v3 T-type calcium channel isoforms at room and physiological temperature. Most of the functional studies regarding T-type channel activity used barium as a charge carrier but it is well known that barium can change the characteristics of the T-type currents (Perez-Reyes, 2003). For this reason we conducted our experiments in the same expression system and under conditions of ionic balance and extracellular calcium levels inherent to physiological conditions. Our recordings indicate several differences between Ca_v3 isoforms at the two temperatures that can affect their contribution to cell function at physiological temperatures.

The relationship between temperature and its effect on channel characteristics is commonly described by the multiplicative constant Q_{10}, which is proportional to the activation energy. However, in the case of T-type calcium channels the effects of temperature have been found to be nonlinear (Narahashi et al., 1987; Rosen, 1996), and thus it is not appropriate to define a firm Q_{10} value based on the two temperatures examined in our study. Rather, we chose to compare channel activities between the room temperature condition most often used in expression system studies to that of a physiological temperature where the functional outcome of channel kinetics is usually interpreted.

Room temperature recordings revealed marked differences between the three T-type isoforms for all biophysical properties examined, with values similar to those reported in previous studies (Cribbs et al., 1998; Klöckner et al., 1999; Lee et al., 1999; McRory et al., 2001; Monteil et al., 2000; Perez-Reyes, 2003). However, we note that our rat $Ca_v3.3$ channel activated at ~25 mV more hyperpolarized potentials than the rat $Ca_v3.3$ variant reported by Lee et al. (1999) and Murbartian et al. (2002). One potential source for this observed difference could be the composition of internal and external recording solutions used in various studies. For example, we do not routinely include ATP and GTP in the patch pipette, but we confirmed in a subset of experiments that inclusion of 2 mM ATP and 0.6 mM GTP in the internal recording solution did not affect the voltage dependence of activation or inactivation for the three rat channel subtypes examined (data not shown). Second, we used intracellular EGTA concentrations that mimic the endogenous calcium buffering capabilities of neurons (Gall et al., 2005). Third, external solutions used to examine T-type channel activity frequently include high concentrations of TEA in the pipette and in the bath, which can affect calcium channel gating (i.e. Chemin et al., 2000; Lee et al., 1999; Murbartian et al., 2002; Stotz et al., 2000). Indeed, as shown in Fig. 5, replacement of CsCl with an essentially equimolar concentration of TEA–Cl (130 mM) shifted the half-activation potential of our rat $Ca_v3.3$ channel toward more depolarized potentials by ~11 mV (from -71.4 ± 1.2 mV to -60.5 ± 1.8 mV, $P=0.01$). Furthermore, the deactivation time constants were dramatically accelerated when TEA was included in the external recording solution (not shown), and these effects were augmented when even higher TEA concentrations were used. Interestingly, this phenomenon was not observed with $Ca_v3.1$, $Ca_v3.2$, or $Ca_v3.3h$ isoforms, indicating that it occurs specifically with our rat $Ca_v3.3$ splice isoform. Hence, we can attribute the observed differences to previously published data at least in part to recording conditions.

On the other hand, the rat $Ca_v3.3$ isoform examined by Lee et al. (1999) and Murbartian et al. (2002) appears to show a more depolarized activation range even in the absence of TEA (see Klöckner et al., 1999). As outlined in the Experimental Procedures section, the rat $Ca_v3.3$ variant used in our study shows several sequence differences compared with that of Lee et al. (1999) and Murbartian et al. (2002), which may contribute to its unique gating characteristics and our observed TEA sensitivity. Taken together, our data suggest that amino acid differences among different types of rat $Ca_v3.3$ constructs (and between the rat and human isoforms; see Experimental Procedures section) in conjunction with particular experimental recording conditions have the propensity to drastically affect the biophysical characteristics of these channels.

Time constants for activation, inactivation, and recovery from inactivation all decreased at physiological temperature, which is consistent with lowered energy barriers during the individual gating transitions. While the relative differences in activation, inactivation and deactivation kinetics among the three channel subtypes were maintained at 37 °C, the kinetics for recovery from inactivation became remarkably similar for all three Ca_v3 family members. This is important, as recovery from inactivation is a key determinant of the ability of T-type channels to trigger rebound burst discharge, follow repetitive activity in neurons, or for the pacemaker current in the heart (Aizenman and Linden, 1999; Perez-Reyes, 2003). Our findings also suggest that all three channel subtypes can be effectively recruited by membrane hyperpolarization at physiological temperatures to contribute to rebound discharge. In the voltage range examined, the time constants of deactivation for $Ca_v3.2$ were not affected by temperature, those of $Ca_v3.1$ were only significantly affected near neuronal resting potential, and those of rat and human $Ca_v3.3$ were significantly decreased but only at potentials more positive than -100 mV. It has been shown that T-type calcium channels can exert significant influence on spike discharge due to their tail currents (Jung et al., 2001; Swensen and Bean, 2003). The temperature dependence of rat $Ca_v3.3$ deactivation implies a greater contribution to calcium influx during inter-spike intervals at lower temperatures. We note that the relative difference in deactivation rates apparent at room temperature between $Ca_v3.3$ and the other isoforms remains at physiological temperature. However, at 37 °C the rate is sufficiently fast as to have significant implications as to the interspike intervals at which deactivation can lead to a cumulative change in membrane depolarization during repetitive activity.

The voltage-dependence of activation and inactivation underwent small changes in response to increasing

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

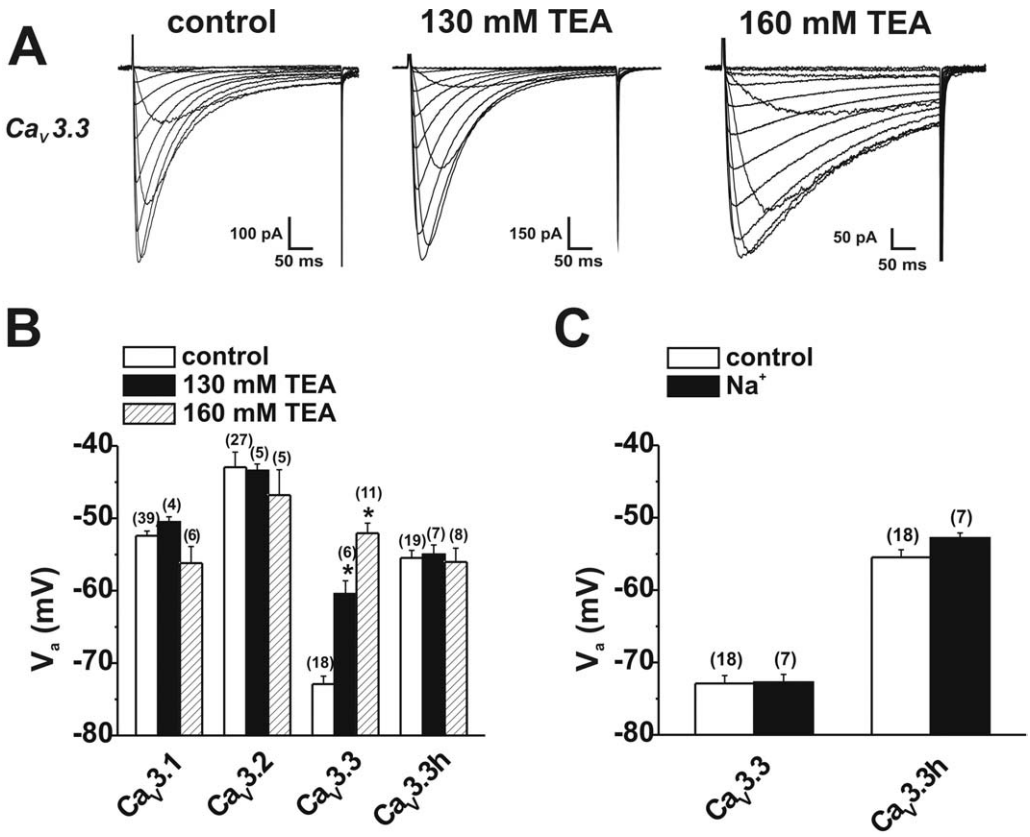

Fig. 5. Effect of TEA on the half activation potential. (A) Representative families of whole-cell currents recorded from tsA-201 cells expressing rat $Ca_v3.3$ calcium channels in the absence and presence of 130 and 160 mM TEA in the extracellular solution, respectively. (B, C) Effects of external TEA and Na ions on the half-activation potentials of the different T-type calcium channel isoforms. The exact composition of the external solutions is described in the Experimental Procedures section. Asterisks denote statistical significance (ANOVA) relative to control solution, and the numbers in parentheses represent the numbers of cells. Note that external TEA causes a selective and statistically significant shift in the half-activation voltage for our rat $Ca_v3.3$ isoform.

temperature that amounted to hyperpolarizing shifts in the window current for $Ca_v3.1$ and $Ca_v3.2$, and a slightly larger window current for rat $Ca_v3.3$ (but not $Ca_v3.3h$). Because the window current occurs in the range of physiological resting membrane potentials, a leftward shift along the voltage axis would imply a significant alteration of the contribution of T-type current to membrane potentials and even basal intracellular calcium levels (Chemin et al., 2000; Huguenard, 1996; Perez-Reyes, 2003). A shift in window current would also be expected to change the dynamics of calcium current activation during the rhythmic interplay between I_h and I_{CaT} currents that underlie oscillatory discharge in thalamic and other neurons (Huguenard and McCormick, 1992).

It has been shown that acidification of the external recording solution (i.e. reduction of pH by as little as 0.25–0.3) induces positive shifts in V_a and V_h, slows deactivation and recovery from inactivation, and can alter peak current amplitude due to proton block, whereas a similar acidification of the internal pH does not appear to affect these channels (Delisle and Satin, 2000; Pinchenko et al., 2005; Tombaugh and Somjen, 1997). As described in the Experimental Procedures section, we noted a decrease in internal and external pH of about 0.1 when out solutions were warmed from room temperatures to 37 °C. This is a much smaller decrease in pH than those shown to mediate major effects on channel gating, although we cannot rule out the possibility that the altered pH may have slightly attenuated the gating effects that occurred after increasing temperatures which were opposite (i.e. hyperpolarizing shifts in V_a and V_h, and accelerated kinetics) to those expected from lowered pH.

An increase in temperature increased the peak current amplitude of all four T-type channel isoforms. Because a change in current amplitude could be measured at both temperatures in the same cells over a short time frame, these changes can be attributed to a change in channel gating rather than variability in expression levels among different cells. Previous studies have also shown that the amount of current is increased when temperature is elevated from ambient to physiological (Coulter et al., 1989; Nobile et al., 1990; Rosen, 1996; Martin et al., 2000), although one study showed that beyond 30 °C current amplitude diminished (Narahashi et al., 1987). The macroscopic current amplitude is determined by the product of the number of channels, the single channel conductance and the open probability. It is unlikely that acute increases in temperature would promote a rapid insertion of new channels into the plasma membrane. Changes in both single channel con-

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

ductance and open probability are both conceivable possibilities to account for the increase in current amplitude, although this discrimination would require a single channel analysis that was not conducted here.

CONCLUSION

In summary, we have shown that the biophysical characteristics of T-type calcium channels are strongly dependent on temperature, with the majority of relative differences in kinetic properties between the isoforms preserved at physiological temperatures and external calcium levels. However, important differences arise in the current kinetics to those reported at room temperature (i.e. rates of inactivation) while other differences are virtually eliminated (i.e. recovery from inactivation). The effects of temperature on the position and magnitude of the window current and rates of deactivation are further expected to influence the activation and role of these isoforms near threshold potential and during repetitive spike discharge. The extent to which these measured parameters can be extrapolated to Ca_v3 channel kinetics *in situ* remains to be determined, but the results indicate the need to take into account the extent to which the properties of these channels change at physiological temperatures when considering their functional roles.

Acknowledgments—We thank Dr. David Parker and Jeanette Mezeyova at NeuroMed Technologies for cDNA sequencing and Dr. Lory for providing human $Ca_v3.3$ cDNA. This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR) to G.W.Z., T.P.S. and R.W.T. G.W.Z. is a Senior Scholar of the Alberta Heritage Foundation for Medical Research (AHFMR). R.W.T. is an AHFMR Scientist. G.W.Z. and T.P.S. are Canada Research Chairs. B.E.M. is supported by CIHR-CGS, Killam Trust and Steinhauer Doctoral Awards.

REFERENCES

Aizenman CD, Linden DJ (1999) Regulation of the rebound depolarization and spontaneous firing patterns of deep nuclear neurons in slices of rat cerebellum. J Neurophysiol 82:1697 1709.

Beedle AM, Hamid J, Zamponi GW (2002) Inhibition of transiently expressed low- and high-voltage-activated calcium channels by trivalent metal cations. J Membr Biol 187:225–238.

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1:11–21.

Cavelier P, Bossu JL (2003) Dendritic low-threshold Ca2+ channels in rat cerebellar Purkinje cells: possible physiological implications. Cerebellum 2:196–205.

Chemin J, Monteil A, Briquaire C, Richard S, Perez-Reyes E, Nargeot J, Lory P (2000) Overexpression of T-type calcium channels in HEK-293 cells increases intracellular calcium without affecting cellular proliferation. FEBS Lett 478:166–172.

Coulter DA, Huguenard JR, Prince DA (1989) Calcium currents in rat thalamocortical relay neurones: kinetic properties of the transient, low-threshold current. J Physiol 414:587–604.

Cribbs LL, Lee JH, Yang J, Satin J, Zhang Y, Daud A, Barclay J, Williamson MP, Fox M, Rees M, Perez-Reyes E (1998) Cloning and characterization of α1H from human heart, a member of the T-type Ca^{2+} channel gene family. Circ Res 83: 103–109.

Delisle BP, Satin J (2000) pH modification of human T-type calcium channel gating. Biophys J 78:1895–1905.

Gall D, Roussel C, Nieus T, Cheron G, Servais L, D'Angelo E, Schiffmann SN (2005) Role of calcium binding proteins in the control of cerebellar granule cell neuronal excitability: experimental and modeling studies. Prog Brain Res 148:321–328.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391:85–100.

Hille B (2001) Ion channels of excitable membranes. Sunderland, MA: Sinauer Associates, Inc.

Huguenard JR (1996) Low-threshold calcium currents in central nervous system neurons. Annu Rev Physiol 58:329–348.

Huguenard JR, McCormick DA (1992) Simulation of the currents involved in rhythmic oscillations in thalamic relay neurons J Neurophysiol 68:1373–1383.

Jung HY, Staff NP, Spruston N (2001) Action potential bursting in subicular pyramidal neurons is driven by a calcium tail current. J Neurosci 21:3312–3321.

Klöckner U, Lee JH, Cribbs LL, Daud A, Hescheler J, Pereverzev A, Perez-Reyes E, Schneider T (1999) Comparison of the Ca^{2+} currents induced by expression of three cloned α1 subunits, α1G, α1H and α1I, of low-voltage-activated T-type Ca^{2+} channels. Eur J Neurosci 11:4171–4178.

Lee JH, Daud AN, Cribbs LL, Lacerda AE, Pereverzev A, Klöckner U, Schneider T, Perez-Reyes E (1999) Cloning and expression of a novel member of the low voltage-activated T-type calcium channel family. J Neurosci 19:1912–1921.

Martin RL, Lee JH, Cribbs LL, Perez-Reyes E, Hanck DA (2000) Mibefradil block of cloned T-type calcium channels. J Pharmacol Exp Ther 295:302–308.

Matteson DR, Armstrong CM (1984) Na and Ca channels in a transformed line of anterior pituitary cells. J Gen Physiol 83:371–394.

McRory JE, Santi CM, Hamming KS, Mezeyova J, Sutton KG, Baillie DL, Stea A, Snutch TP (2001) Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276:3999–4011.

Monteil A, Chemin J, Leuranguer V, Altier C, Mennessier G, Bourinet E, Lory P, Nargeot J (2000) Specific properties of T-type calcium channels generated by the human alpha 1I subunit. J Biol Chem 275:16530–16535.

Murbartian J, Arias JM, Lee JH, Gomora JC, Perez-Reyes E (2002) Alternative splicing of the rat Ca(v)3.3 T-type calcium channel gene produces variants with distinct functional properties (1). FEBS Lett 528:272–278.

Narahashi T, Tsunoo A, Yoshii M (1987) Characterization of two types of calcium channels in mouse neuroblastoma cells. J Physiol 383:231–249.

Nobile M, Carbone E, Lux HD, Zucker H (1990) Temperature sensitivity of Ca currents in chick sensory neurones. Pflugers Arch 415:658–663.

Perez-Reyes E (2003) Molecular physiology of low-voltage-activated T-type calcium channels. Physiol Rev 83:117–161.

Perez-Reyes E, Cribbs LL, Daud A, Lacerda AE, Barclay J, Williamson MP, Fox M, Rees M, Lee JH (1998) Molecular characterization of a neuronal low-voltage-activated T-type calcium channel. Nature 391:896–900.

Pinchenko VO, Kostyuk PG, Kostyuk EP (2005) Influence of external pH on two types of low-voltage-activated calcium currents in primary sensory neurons of rats. Biochim Biophys Acta 1724: 1–7.

Raman IM, Bean BP (1999) Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. J Neurosci 19:1663–1674.

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010

Rosen AD (1996) Temperature modulation of calcium channel function in GH3 cells. Am J Physiol 271:C863–C868.

Stotz SC, Hamid J, Spaetgens RL, Jarvis SE, Zamponi GW (2000) Fast inactivation of voltage-dependent calcium channels. A hinged-lid mechanism? J Biol Chem 275:24575–24582.

Swensen AM, Bean BP (2003) Ionic mechanisms of burst firing in dissociated Purkinje neurons. J Neurosci 23:9650–9663.

Tombaugh GC, Somjen GG (1997) Differential sensitivity to intracellular pH among high- and low-threshold Ca^{2+} currents in isolated rat CA1 neurons. J Neurophysiol 77:639–653.

(Accepted 14 July 2006)

Please cite this article as: M. Iftinca, et al., Temperature dependence of T-type calcium channel gating, Neuroscience (2006), doi: 10.1016/j.neuroscience.2006.07.010