TEMPERATURE DEPENDENCE OF T-TYPE CALCIUM CHANNEL GATING

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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 (CaT) 3.1, CaT 3.2, and CaT 3.3, as well as human CaT 3.3 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 CaT 3.1 and CaT 3.2. At 37 °C the half-inactivation showed a hyperpolarizing shift for CaT 3.1 and CaT 3.2 and human CaT 3.3, but not rat CaT 3.3. 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. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

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 (CaT 3.1, CaT 3.2, and CaT 3.3). 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 GH3 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-
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,3 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,3.1, Ca,3.2 and Ca,3.3 cDNAs were the same as those isolated and described by McRory et al. (2001). Our rat Ca,3.1 sequence (AF29012) is identical to that isolated by Perez-Reyes and colleagues (NM_031601; Perez-Reyes et al., 1998). The rat Ca,3.3 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 III54 region. Because a recent study examining splice variants of Ca,3.3 could not verify the existence of a variant lacking these residues (Murbartian et al., 2002), we re-sequenced the Ca,3.3 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. The Cav 3.3 clone is the "a" splice variant (accession number AF290213). 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,3.3 cDNA (denoted as Ca,3.3h) was as described previously (Monteil et al., 2000).

The Cav 3.3h clone is the "a" splice variant (accession number NP_006919) 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 areas 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% CO2) 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,3.1, 3.2 and 3.3 α1 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 Ca2+ (in mM: 128.25 CsCl, 1.5 CaCl2, 1.5 MgCl2, 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 CaCl2, 1.5 MgCl2, 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 CaCl2, 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,3 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 -- 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 MgCl2, 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 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 3.3h 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.
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:

\[
I = \frac{1}{1 + e^{-(V-V_{1/2})/k}}
\]

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

\[
I = \frac{1}{1 + e^{-\left(V-E_{rev}\right)/k}} \times G \times (V-E_{rev})
\]

where \(E_{rev}\) is the reversal potential and \(G\) is the maximum slope conductance and \(V_a\) the half activation potential.

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

\[
i(t) = I_{peak}(1 - e^{-t/\tau_a})
\]

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

\[
i(t) = I_{peak}(e^{-t/\tau_i})
\]

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_3 calcium channel gating, we exogenously expressed the three different rat brain T-type calcium channel isoforms (Ca_3.1, Ca_3.2, and Ca_3.3, 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_3.1 channels exhibited the fastest gating kinetics followed by Ca_3.2 and Ca_3.3. While the gating characteristics of rat Ca_3.1 and Ca_3.2 were found to be similar to those reported previously by other investigators, the rat Ca_3.3b 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_3.3a isoform (see Murbartian et al., 2002) or with human Ca_3.3 (Monteil et al., 2000). Because of this unique gating behavior of the rat Ca_3.3 construct, we conducted a series of parallel experiments using Ca_3.3h (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_3.3 isoform are not due to problems in recording Ca_3.3 channel activity under our experimental conditions. Instead, as we will outline below, the specific properties observed with rat Ca_3.3 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_3 channel subtypes as well as the Ca_3.3h 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_3.1 (n=14, \(P=0.002\)), 2.1-fold for Ca_3.2 (n=11, \(P=0.001\)) and 1.6-fold for Ca_3.3 (n=11, \(P=0.002\); paired t-test). Similar results were obtained with Ca_3.3h (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_3.1 and Ca_3.2 channels upon increasing the temperature (\(P<0.04\), Q=1.15 and 1.2, respectively), whereas that of rat Ca_3.3 or Ca_3.3h 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_3.1, Ca_3.2, and Ca_3.3h and a small depolarizing shift for the rat Ca_3.3 (Fig. 2A, Table 1); effects that were statistically significant only for the Ca_3.2 subtype (\(P=0.04\), Q=1.15) and Ca_3.3h (\(P=0.01\), 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_3.1 and Ca_3.2 (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_3.1 and Ca_3.3, but was observed only below −20 mV for Ca_3.2 (Fig. 2C). It should be noted that the time course of inactivation was adequately described by a
Table 1. Effect of temperature on the half activation ($V_{a}$) and half inactivation ($V_{h}$) potentials of Ca3 isoforms

<table>
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<tr>
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<th>Ca3.1</th>
<th>21°C</th>
<th>37°C</th>
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<td>$V_{a}$ (mV)</td>
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<td>Q</td>
<td>5.4±0.3</td>
<td>4.2±1.1</td>
<td>6.2±0.9</td>
<td>0.78</td>
<td>1.15</td>
<td>0.78</td>
<td>1.54</td>
<td>1.15</td>
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<td>$k_{activ}$</td>
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<td>Q</td>
<td>0.89</td>
<td>0.01</td>
<td>0.04</td>
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<td><strong>Inactivation</strong></td>
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<td>$V_{h}$ (mV)</td>
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<td>Q</td>
<td>1.06</td>
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<td>Q</td>
<td>0.07</td>
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<td><strong>Deactivation</strong></td>
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<td>$\tau_{deactivation}$ (ms)</td>
<td>1.3±0.1 (6)</td>
<td>1.5±0.2 (6)</td>
<td>1.2±1.0 (6)</td>
<td>1.0±0.2 (4)</td>
<td>1.8±0.5 (5)</td>
<td>1.1±0.0 (4)</td>
<td>1.9±0.1 (13)</td>
<td>0.8±0.1 (12)*</td>
<td>0.42</td>
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<td>Q</td>
<td>1.15</td>
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<td><strong>Recovery</strong></td>
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<td>$\tau_{recovery}$ (ms)</td>
<td>310.1±4.3 (10)</td>
<td>123.6±24.9 (8)*</td>
<td>527.8±34.5 (8)</td>
<td>184.1±31.9 (6)*</td>
<td>615.2±86.2 (11)</td>
<td>205.3±3.6 (6)*</td>
<td>620.4±52.9 (8)</td>
<td>183.8±1.8 (8)*</td>
<td>0.3</td>
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<tr>
<td>Q</td>
<td>0.4</td>
<td>0.35</td>
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$k_{activ}$ 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).
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 Cav3.1 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 Cav3.3 (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, Cav3.3 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
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 \(/-110\) mV for Cav3.1 (\(n=10\) and 8, respectively, \(P=0.02, Q=0.4\), Cav3.2 (\(n=8\) and 6, respectively, \(P=0.01, Q=0.35\)), and rat Cav3.3 (\(n=11\) and 6, respectively, \(P=0.01, Q=0.33\)) (Fig. 3A–C, Table 1). Analogous results were obtained with the Cav3.3 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 Cav3.1 and Cav3.2; and \(-90\) and \(-110\) mV for Cav3.3). 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 Cav3.2 recovered from inactivation more slowly than Cav3.1 (Cav3.1: at 21 °C: 444.1±76.2 ms (\(n=5\)) and at 37 °C: 197.3±15.5 ms (\(n=5, P=0.02, Q=0.44\)), Cav3.2: 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 Cav3.3 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
Time constants were similar at both temperatures for Cav3.2 regardless of the membrane potentials tested (Fig. 4C, Table 1), whereas they became significantly different at more depolarized potentials for Cav3.1 and rat Cav3.3 (Fig. 4B, D). Interestingly, at both temperatures the rat and human Cav3.3 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 Cav3.3 was substantially
slower to deactivate at typical neuronal resting potentials. Moreover, the values obtained for Cav3.3h channels were smaller than those seen with the rat Cav3.3 isoforms, but still showed temperature dependence (Q = 1.15 for Cav3.1; Q = 0.83 for Cav3.2; Q = 0.56 for Cav3.3; and Q = 0.42 for Cav3.3h) (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; Noble et al., 1990; Rosen, 1996). Because these preparations...
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$_{3.3}$ 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$_{3.3}$ 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$_{3.3}$ channel activated at $-25$ mV more hyperpolarized potentials than the rat Ca$_{3.3}$ 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–Cl (130 mM) shifted the half-activation potential of our rat Ca$_{3.3}$ channel toward more depolarized potentials by $-11$ mV (from $-71.4 \pm 1.2$ mV to $-60.5 \pm 1.8$ 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$_{3.1}$, Ca$_{3.2}$, or Ca$_{3.3h}$ isoforms, indicating that it occurs specifically with our rat Ca$_{3.3}$ 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$_{3.3}$ 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$_{3.3}$ 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$_{3.3}$ 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$_{3.3}$ 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$_{3.3}$ were not affected by temperature, those of Ca$_{3.1}$ were only significantly affected near neuronal resting potential, and those of rat and human Ca$_{3.3}$ 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$_{3.3}$ 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$_{3.3}$ 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
temperature that amounted to hyperpolarizing shifts in the window current for CaV3.1 and CaV3.2, and a slightly larger window current for rat CaV3.3 (but not CaV3.3h). 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 Ih and ICaT 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 VA and Vh, 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 (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 Ih and ICaT currents that underlie oscillatory discharge in thalamic and other neurons (Huguenard and McCormick, 1992).

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-
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