In Vitro Characterization of T-Type Calcium Channel Antagonist TTA-A2 and In Vivo Effects on Arousal in Mice

Richard L. Kraus, Yuxing Li, Yun Gregan, Anthony L. Gotter, Victor N. Uebele, Steven V. Fox, Scott M. Doran, James C. Barrow, Zhi-Qiang Yang, Thomas S. Reger, Kenneth S. Koblan, and John J. Renger


Received June 4, 2010; accepted August 3, 2010

ABSTRACT

T-type calcium channels have been implicated in many behaviorally important neurophysiological processes, and altered channel activity has been linked to the pathophysiology of neurological disorders such as insomnia, epilepsy, Parkinson’s disease, depression, schizophrenia, and pain. We have previously identified a number of potent and selective T-type channel antagonists (Barrow et al., 2007; Shipe et al., 2008; Yang et al., 2008). Here we describe the properties of the antagonist TTA-A2 [2-(4-cyclopropylphenyl)-N-((1R)-1-{5-[(2,2,2-trifluoroethyl)oxo]-pyridin-2-yl}ethyl)acetamide], assessed in patch-clamp experiments. TTA-A2 blocks T-type channels (Cav3.1, Cav3.2, Cav3.3) voltage dependently and with high potency (IC50 ~100 nM). Stimulation at 3 Hz revealed additional use dependence of inhibition. A hyperpolarized shift of the channel availability curve and delayed channel recovery from inactivation suggest that the compound preferentially interacts with and stabilizes inactivated channels. The compound showed a ~300-fold selectivity for Cav3 channels over high-voltage activated calcium channels. Inhibitory effects on native T-type currents were confirmed in brain slice recordings from the dorsal lateral geniculate nucleus and the subthalamic nucleus. Furthermore, we demonstrate that in vivo T-type channel inhibition by TTA-A2 suppresses active wake and promotes slow-wave sleep in wild-type mice but not in mice lacking both Cav3.1 and Cav3.3, suggesting the selective effect of TTA-A2 on recurrent thalamocortical network activity. The discovery of the potent and selective T-type channel antagonist TTA-A2 has enabled us to study the in vivo effects of pharmacological T-channel inhibition on arousal in mice, and it will help to explore the validity of these channels as potential drug targets for sleep-related and other neurological diseases.

Introduction

Calcium, the most common signal transduction element in cells, enters the cytosol through highly selective voltage-gated calcium channels. Electrophysiological studies have identified two major classes of calcium channels, high-voltage-activated (L-type: Cav1.1, Cav1.2, Cav1.3, Cav1.4; P/Q-type: Cav2.1) N-type: Cav2.2, R-type: Cav2.3) and low-voltage-activated (T-type: Cav3) calcium channels. In contrast to high-voltage-activated calcium channels, T-type channels were defined by activation at depolarizations near the resting membrane potential, fast gating kinetics, slow deactivation kinetics, and small single channel conductance (Catterall et al., 2005). To date, three T-type channels, Cav3.1, Cav3.2, and Cav3.3, have been cloned in mice, rats, and humans, showing all the properties of native neuronal T-type calcium channels when expressed heterologously (Perez-Reyes, 2003).

Low-voltage-activated T-type calcium channels are expressed throughout the body. They have been implicated in many physiological processes as diverse as hormone secretion, smooth muscle contraction, fertilization, cell growth and proliferation, pacemaker activity, pain processing (Perez-Reyes, 2003; Zamponi et al., 2009), low-threshold spikes (LTS), and thalamocortical rhythms during sleep and epilepsy (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009). Calcium-dependent low-threshold currents mediated by T-type calcium channels underlie low-threshold spikes that have been demonstrated as intrinsic firing properties of thalamocortical neurons in vivo (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006; Beenhakker and Huguenard, 2009).
lamic and cortical neurons producing network oscillations and generating characteristic EEG patterns during wakefulness, non-REM sleep (delta slow-wave sleep), and REM sleep. LTS are predominately observed in the thalamus during non-REM sleep and are generally absent during wakefulness and REM sleep, suggesting a key role of T-type calcium channels in the regulation of sleep (Steriade et al., 1993; McCormick and Bal, 1997; Crunelli et al., 2006).

Abnormal neuronal hyperpolarization that results in aberrant thalamocortical oscillations that depend on T-type channel activity has been linked to the pathophysiology of a variety of neurological disorders. It is believed that dysfunction at the level of thalamic relay cells induces thalamocortical dysrhythmia linked to insomnia, absence epilepsy, Parkinson’s disease, tinnitus, depression, schizophrenia, and neurogenic pain (Huguenard and Prince, 1994; Huguenard, 1999; Llinás et al., 1999). Consistently, data from Ca3 transgenic, gene knockout, and knockdown animal models suggest the involvement of T-type channels in epilepsy, pain signaling (Kim et al., 2001, 2003; Bourinet et al., 2005; Choi et al., 2007), and sleep regulation (Kim et al., 2001; Lee et al., 2004; Anderson et al., 2005). A recent study has also suggested a role of T-type channels in body weight maintenance (Uebele et al., 2009a).

Many drugs in clinical use, including antipsychotic (penfluridol, clozapine, haloperidol, pimozide), antidepressants (fluoxetine, trazodone), and antiepileptics (ethosuximide, phenytoin, zonisamide), have been shown to have activity on T-type channels (Heady et al., 2001; Santi et al., 2002; Traboulse et al., 2006; Kraus et al., 2007). Although most of those molecules are neither selective nor potent on T-type channels, their activity provides evidence for the potential of T-type channels as therapeutic targets (Shin et al., 2008). Mibebradil (Posicor), the first T-type calcium channel antagonist identified, was introduced to the clinic as an antihypertensive, and later withdrawn from the U.S. market because of interactions with liver enzymes (Van der Vring et al., 1999). Despite its activity on other high-voltage-activated ion channels, mibebradil is frequently used as a pharmacological research tool to study T-type channels (Mehrke et al., 1994; Heady et al., 2001; Leurraguer et al., 2001). To more convincingly understand the true role of T-type channels, potent and selective agents are needed to study T-type channel function in neurological disease states and to validate T-type channels as efficacious drug targets. Consequently, numerous efforts to identify such T-type channel antagonists have been initiated in several laboratories. The discovery of a number of potent and selective T-type calcium channel antagonists has recently been reported (Barrow et al., 2007; Shipe et al., 2008; Yang et al., 2008; Uebele et al., 2009b).

Here, we describe the activity of the pyridyl amide T-type calcium channel antagonist TTA-A2 (2-(4-cyclopentyl-1-(4-((2,2,2-trifluoroethyl)oxo)pyridin-2-yl)ethyl) acetamide) on recombinant human T-type calcium channels expressed in HEK-293 cells, including its potency, voltage and use dependence, and effects on channel gating. In addition, we confirmed compound activity on native T-type currents from thalamic relay neurons in the dorsal lateral geniculate nucleus (dLGN) and the subthalamic nucleus (STN), and demonstrate T-type channel-selective pharmacodynamic effects of TTA-A2 in vivo.

### Materials and Methods

#### Low Voltage-Activated T-Type Calcium Channels

For whole-cell voltage-clamp recordings, cell lines expressing human Ca3.1, Ca3.2, or Ca3.3 channels were used. Tetracaine-inducible HEK-293 cells that stably express the tetracaine repressor protein were transfected with Ca3.1, Ca3.2, and Ca3.3 in pcDNA 4/TO. Cells were maintained in DMEM (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (Clontech, Mountain View, CA; 2 mM L-glutamine, 100 units/ml penicillin/streptomycin (Invitrogen), 150 μg/ml zeocin (phleomycin; Invitrogen), and 5 μg/ml blasticidin (Invitrogen). Cells were plated on poly-D-lysine-coated cover slips, and Ca3.3 channel expression was induced by exposure to 0.1 mM tetracaine (Sigma-Aldrich, St. Louis, MO) for at least 24 h before recording. Cells were then washed with mammalian Ringer’s solution immediately before recording.

#### High Voltage-Activated Calcium Channels

L-type currents (Ca1.2) were recorded from HEK-293 cell coexpressing α1C, β2, δ, and Kir2.3 (Xia et al., 2004). Cells were grown in DMEM (Invitrogen) and supplemented with 10% fetal calf serum (Sigma F-2442), 40 μg/ml phleomycin, 100 μg/ml hygromycin (Invitrogen), and 10 μg/ml G-418 (Invitrogen). P-type currents (Ca2.1) were recorded from HEK-293 cells coexpressing α1A, β2, δ, and Kir2.3. Cells were grown in DMEM, 10% fetal bovine serum, penicillin/streptomycin, Geneticin (G-418; Invitrogen), phleomycin, and 6 μg/ml blasticidin. R-type currents (Ca2.2) were recorded from HEK-293 cells coexpressing α1B, β2, δ, and Kir2.3. Cells were grown in DMEM, 10% fetal bovine serum, penicillin/streptomycin, Geneticin (G-418; Invitrogen), phleomycin, and 10 μg/ml blasticidin. R-type currents (Ca2.3, 2.4) were recorded from HEK-293 cells coexpressing α1E, β2, δ, and Kir2.3. Cells were grown in DMEM, 10% fetal bovine serum, penicillin/streptomycin, Geneticin (G-418, 418; Invitrogen), phleomycin, and 6 μg/ml blasticidin. All cell lines were maintained at 5% CO2 at 37°C (Dai et al., 2008). Ca2.1 expressing cell line was transferred to 30°C/5% CO2 for 48 h after cells achieved 80% confluence. Cells were plated on poly-D-lysine-coated cover slips for at least 24 h before recording.

#### Recombinant Cell Electrophysiology

Single-cell recordings of currents through low-voltage-activated Ca3.1, Ca3.2, and Ca3.3 calcium channels and high-voltage-activated Ca1.2, 1.2A, 1.2B, and Ca2.3 calcium channels were performed at room temperature (20–22°C) using the whole-cell patch-clamp technique. Currents were recorded using a HEKA EPC-9 patch-clamp amplifier. Data were stored on a personal computer equipped with HEKA Pulse 8.5 and analyzed using Pulsefit and FitMaster (HEKA, Lambrecht/Pfalz, Germany), Igor Pro 4.0 (Wavemetrics, Lake Oswego, OR), Origin 6.0 (MicroCal LLC, Northampton, MA), and Prism 4 (GraphPad Software Inc., San Diego, CA). Patch pipettes were made from borosilicate glass tubing (World Precision Instruments, Inc., Sarasota, FL), fire polished, and had a resistance of 1 to 4.5 MΩ when filled with internal solution measured in the recording medium. Voltage errors were minimized by series resistance compensation (typically 70%). Leak resistance and capacitance were corrected by subtracting the scaled current observed with a P/4 protocol. Data were acquired at 10 to 50 kHz and filtered at 5 to 10 kHz. Salts for recording solutions were purchased from Sigma-Aldrich. For T-type calcium current measurements, the following solutions were used. Pipette solution consisted of 125 mM CsCl, 10 mM TEACI4, 10 mM HEPES, 8 mM NaCl, 0.06 mM CaCl2, 0.6 mM EGTA, 4 mM ATP-Mg, 0.3 mM GTP, and 280 mM, pH 7.2, adjusted with CsOH. The bath solution consisted of 130 mM NaCl, 30 mM d(+)-glucose, 20 mM HEPES, 4 mM KCl, 2 mM CaCl2, and 1 mM MgCl2, pH 7.4. For high-voltage-activated channel recordings, the internal solution was 135 mM CsCl, 10 mM HEPES, 1 mM MgCl2, 10 mM EGTA, 5 mM ATP-Mg, and 0.1 mM GTP; pH 7.2. The bath solution was 150 mM Chol-Cl, 10 mM HEPES, 1 mM MgCl2, 15
Brain Slice Preparation

All animals used in these studies were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Merck Research Laboratories Institutional Animal Care and Use Committee approved all studies described in this manuscript, and experimental protocols were in accordance with all applicable guidelines regarding the care and use of animals. Animals were housed in a Association for Assessment and Accreditation of Laboratory Animal Care International-approved facility with free access to food and water. Electrophysiology experiments were performed on normal sagittal slices from 15- to 20-day-old Sprague-Dawley rats (Taconic Farms, Germantown, NY). Animals were decapitated and brains were rapidly removed and submerged in an ice-cold choline replacement solution (CRS) containing 126 mM choline chloride, 5 mM KCl, 1.2 mM NaH2PO4, 1.3 mM MgCl2, 8 mM MgSO4, 10 mM glucose, and 26 mM NaHCO3, equilibrated with 95% O2/5% CO2. Slices 350 μm-thick were obtained using a vibrating blade microtome (Leica Microsystems, Inc., Deerfield, IL). Slices were immediately transferred to a holding chamber containing normal artificial cerebrospinal fluid (aCSF); 124 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 1.0 mM NaH2PO4, 2 mM CaCl2, 20 mM glucose, and 26 mM NaHCO3, equilibrated with 95% O2/5% CO2 that was maintained at room temperature. In all experiments, 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenic acid were included in the choline chloride buffer and in the holding chamber aCSF.

Brain Slice Recordings

Whole-cell patch clamp recordings were performed from thalamic neurons in 350 μm-thick midbrain slices at a temperature of 32°C. During recordings, slices were maintained fully submerged and continuously perfused (2–3 ml/min) with 95% O2/5% CO2 equilibrated aCSF. Cells in the subthalamic nucleus and the dorsal lateral geniculate nucleus were visualized using a differential interference contrast microscope and an infrared video system. Salts for recording solutions and tetrodotoxin were purchased from Sigma-Aldrich. Recording electrodes were pulled from borosilicate glass (Leica Microsystems, Inc., Deerfield, IL). Slices were immediately transferred to a holding chamber containing normal artificial cerebrospinal fluid (aCSF); 124 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 1.0 mM NaH2PO4, 2 mM CaCl2, 20 mM glucose, and 26 mM NaHCO3, equilibrated with 95% O2/5% CO2 that was maintained at room temperature. In all experiments, 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenic acid were included in the choline chloride buffer and in the holding chamber aCSF.

Electrophysiology Analysis

Concentration-response curves were fitted using a sigmoidal Hill function, \( I = I_{\max} \frac{1+(EC_{50} - V)/hill\ slope}{1+(EC_{50} - V)/hill\ slope} \). 95% Confidence intervals are listed for IC50 values and Hill coefficients. Channel activation and inactivation curves resulting from the voltage command protocols were plotted to the Boltzmann equation: \( I/I_{\max} = 1/(1+exp(V_{0.5}-V/k)) \) to derive half-maximal voltage (V0.5) and slope factor (k) for comparison. Time course of recovery from inactivation was best fitted by a single exponential decay-yielding time constant (τ) in the absence and presence of drug. Student’s t test, ANOVA one-way post hoc Dunnett’s test, was used for statistical analysis, and values were considered significant at \( p < 0.05 \). Data are given as mean ± S.E.M.

Mouse Polysomnography

Mice homozygous for targeted mutation of both Cacna1g (Ca3.1) and Cacna1l (Ca3.3) genes along with wild-type controls were generated on a C57BL6/Sv129 background and bred identically. Heterozygous Cacna1g and Cacna1l mutant lines obtained from Deltagen were intercrossed at Taconic Farms to generate double mutant homozygous mice, as well as wild-type animals. Before the study, animals were acclimated to a 12:12-light/dark schedule (lights on at 2:00 PM, lights off at 2:00 AM). All animal procedures were conducted in accordance with Merck Institutional Animal Care and Use Committee.

Double Ca3.1/Ca3.3 knockout (n = 6) and wild-type (n = 6) mice were surgically implanted with TL11M2-F20-EET radio telemetry transmitters (Data Sciences International [DSI], St. Paul, MN). Electrocardiogram (ECOG) electrode placement includes one electrode positioned 1 mm anterior to bregma, 2 mm lateral of the central suture, and a second at 1 mm anterior to lambda and 2.5 mm lateral fixed in predrilled holes with stainless steel screws. Electromyogram (EMG) electrodes were placed below the trapezius muscle. Radiotelemetry ECOG and EMG signals were acquired at a 500 Hz rate from RPC-1 receivers using DataQuest A.R.T. 3.1 Acquisition software (DSI). Automated raw data upload was performed daily to a remote server, and sleep scoring was performed using mouse scoring parameters on the Somnologica Data analysis package (Embla, ON, Canada). Surgical procedures and the accuracy of automated sleep scoring parameters have been confirmed by hand scoring of ECOG and EMG traces. The effects of 10 mg/kg TTA-A2 was assessed in both wild-type and double Ca3.1/Ca3.3 knockout animals in a 5-day crossover dosing paradigm in which animals received a once-daily treatment 1 h before lights on (ZT 23:00; 13:00 DST). Daily vehicle and compound treatments occurred for the first 5 days, followed by 2 days of washout and subsequently a reversal of the vehicle and compound treatment groups, such that all animals ultimately received both vehicle and compound. The time spent in active wake, REM, and delta (slow-wave) sleep for each 30-min period from each animal for all 5 days of the treatment arm was averaged according to the treatment condition and was plotted over a single 24-h period. Significant differences between vehicle and compound treatment groups at each 30-min interval were determined using the linear effects model for repeated measures.

Results

The mechanism of T-type calcium channel block by TTA-A2 (chemical structure shown in Fig. 1) was studied in whole-cell patch clamp experiments using three stable HEK-293 cell lines expressing human Ca3.1, Ca3.2, or Ca3.3 channels.

First we assessed Ca3.1 channel activity (as previously reported in Uebele et al., 2009a). To evoke maximal Ca3.1 currents, 30-ms test pulses to the peak of the I/V curve, −20 mV, were given every 10 s from a holding potential of −100 mV. TTA-A2 caused a concentration-dependent decrease of the measured Ca3.1 peak current amplitude. Inhibition was reversible upon washout. Normalized steady-state current amplitudes in the presence of drug were plotted against drug concentrations, and the resulting curve was fitted to the Hill equation. Under these experimental conditions, the concen-
tation required for 50% current inhibition (IC_{50}) at −100 mV was 4.1 μM (Fig. 2B).

According to the modulated receptor hypothesis, drugs can have varying affinities for channels in the closed/resting, open, and inactivated state (Hille, 1977; Hondeghem and Katzung, 1984). Based on T-type calcium channel availabil-

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** Inhibition of Cav3 T-type calcium channels by TTA-A2. A, top, Cav3.1 current traces evoked by 30 ms depolarizations steps from −100 to −20 mV under control conditions and at steady-state in the presence of 1, 3, and 10 μM TTA-A2. A, bottom, Cav3.1 current traces evoked by 30-ms depolarizations steps from −80 to −20 mV under control conditions and at steady-state in the presence of 30, 100, and 300 nM TTA-A2 (Uebele et al., 2009a). B, concentration-response curve of TTA-A2 for Cav3.1 channels at holding potentials of −100 and −80 mV. Data points reflect mean ± S.E. of three to four determinations. Error bars are hidden behind symbols. IC_{50} values of 4.1 μM (95% CI 3.7 to 4.4 μM) and 89 nM (95% CI 77 to 102 nM) and Hill coefficients of 1.2 (95% CI 1.3 to 1.1) and 1.4 (95% CI 1.7 to 1.1) were determined at −100 and −80 mV, respectively, using the equation of a sigmoidal dose-response curve (Uebele et al., 2009a). C, top, Cav3.2 current traces evoked by 30-ms depolarizations steps from −100 to −20 mV under control conditions and in the presence of 1, 3, and 10 μM TTA-A2. C, bottom, Cav3.2 current traces evoked by 30-ms depolarizations steps from −80 to −20 mV under control conditions and in the presence of 30, 100, and 300 nM TTA-A2. D, concentration-response curve of TTA-A2 for Cav3.2 channels at holding potentials of −100 and −80 mV. Data points reflect mean ± S.E. of three to four determinations. IC_{50} values of 5.6 μM (95% CI 3 to 10 μM) and 92 nM (95% CI 67 to 128 nM) and Hill coefficients of 1.1 (95% CI 2 to 0.3) and 1.4 (95% CI 1.6 to 0.7) were determined at −100 and −80 mV, respectively, using the equation of a sigmoidal dose-response curve. E, top, Cav3.3 current traces evoked by 30-ms depolarizations steps from −100 to −20 mV under control conditions and in the presence of 1, 3, and 10 μM TTA-A2. E, bottom, Cav3.3 current traces evoked by 70-ms depolarizations steps from −80 to −20 mV under control conditions and in the presence of 30, 100, and 300 nM TTA-A2. F, concentration-response curve of TTA-A2 for Cav3.3 channels at holding potentials of −100 and −80 mV. Data points reflect mean ± S.E.M. of three to nine determinations. Error bars are hidden behind symbols. IC_{50} values of 3.7 μM (95% CI 3.1 to 4.3 μM) and 98 nM (95% CI 83 to 114 nM) and Hill coefficients of 1.2 (95% CI 1.5 to 1.0) and 1.4 (95% CI 1.3 to 0.9) were determined at −100 and −80 mV, respectively, using the equation of a sigmoidal dose-response curve.
ity curves, at negative membrane potentials, such as −100 mV, most of the channels are in the closed/resting state, whereas a larger fraction of channels resides in the inactivated state at a more depolarized potential of −80 mV. TTA-A2 showed increased affinity for Ca₃.1 channels at this more depolarized potential (IC₅₀ = 89 nM), indicating that block was state-dependent. Time course of block was similar at the two holding potentials tested. Recovery from block at −80 mV was slower upon washout. TTA-A2 did not change the time course of macroscopic Ca₃.1 current activation and inactivation kinetics during applied test pulses (Fig. 2A). Scaled currents remaining in the presence of drug showed an essentially identical time course compared with untreated current traces (not shown).

To assess selectivity among Ca₃ subtypes, TTA-A2 block of Ca₃.2 and Ca₃.3 channels was studied. Potency on Ca₃.2 was comparable and also voltage-dependent. IC₅₀ values of 5.6 µM and 92 nM were measured at −100 and −80 mV, respectively (Fig. 2, C and D). Ca₃.3 channels show slower activation and inactivation kinetics than Ca₃.1 and Ca₃.2 channels. To capture evoked peak current amplitudes and to allow for complete recovery from inactivation between depolarizations, 70-ms test pulses to −20 mV were given every 20 s. TTA-A2 also inhibited Ca₃.3 currents voltage-dependently, yielding similar IC₅₀ values of 3.7 µM and 98 nM at −100 and −80 mV, respectively (Fig. 2, E and F). The Ca₃.1 channel cell line was selected for robust current recordings with minimal current rundown during longer experiments to study the mechanism of T-channel block by TTA-A2 in more detail.

First we analyzed compound effects on Ca₃.1 channel gating. T-type calcium currents deactivate slowly in a voltage-dependent manner, resulting in slowly decaying tail currents during a repolarization phase after the test pulse. Ca₃.1 tail current amplitude at −80 mV was measured after brief depolarizations (8 ms) to different test pulses from −80 to +50 mV in the absence or presence of TTA-A2 and plotted against test pulse potential. Activation curves were well described by a single Boltzmann function (Fig. 3A). TTA-A2 (300 nm) caused robust current inhibition at −80 mV (82.7 ± 1.8%, n = 3) but did not significantly shift midpoint of the Ca₃.1 activation curve (V₀.5act-control = −40.5 ± 1.2 mV, k = 7.4 ± 0.7, and V₀.5act-300 nM TTA-A2 = −43.9 ± 1.9 mV, k = 5.2 ± 0.6; n = 4–6), indicating that the drug did not interfere with channel activation kinetics.

Changes in Ca₃ current inhibition as a function of the holding potential suggested that TTA-A2 block was state-dependent and that compound may preferentially interact with inactivated channels. Drugs that bind and stabilize the inactivated state of voltage-gated channels shift the equilibrium from closed/resting to inactivated states and consequently induce a shift of the steady-state inactivation curve to more negative potentials. Channel availability curves in the absence of TTA-A2 and after bath equilibration with compound were established using tail current analysis. Following 10-s prepulses to potentials ranging from −120 to −35 mV and a subsequent 8-ms test pulse to −20 mV, tail current amplitudes were measured at −80 mV and plotted against prepulse potential. The resulting inactivation curves were fitted with the Boltzmann equation. Under the experimental conditions used, the midpoint (V₀.5inact) of channel inactivation was shifted in a concentration-dependent manner to hyperpolarized potentials by 7 ± 0.8 and 13.8 ± 1.2 mV at 100 nM and 1 µM TTA-A2, respectively (Fig. 3B).
Next, we investigated the effect of TTA-A2 on the time course of Cav3.1 channel recovery from fast inactivation, defined as calcium current recovery within 5 s. Using a standard double-pulse protocol, small current amplitudes and slow channel recovery between double pulses did not allow accurate measurement of compound effects on recovery from inactivation at −80 mV. Consequently, cells were held at −100 mV to obtain robust currents and enable maximal channel recovery within 5 s. Following a 100-ms conditional prepulse to −20 mV, the time course of current recovery at −100 mV was determined by applying 100-ms test pulses to −20 mV at various time intervals after the prepulse. Cav3.1 channels were allowed to recover at −100 mV for 15 s in between sweeps. Peak calcium currents evoked by the test pulse were normalized to the peak current amplitudes measured during the prepulse and plotted against time. As shown in Fig. 3C, Cav3.1 current recovery at −100 mV was well described by a single exponential. A concentration of 5 μM TTA-A2, which caused approximately 50% current inhibition at −100 mV, significantly delayed recovery from inactivation, evident as a 3.5-fold increase of the recovery time constant (τrec = 213 ± 14 ms; τrec,0M TTA-A2 = 764 ± 44 ms; n = 10–21).

More frequent stimulations favor the availability of open and/or inactivated channels, thereby increasing the affinity of state-dependent blockers to their target. We tested to determine whether TTA-A2 exerts a use-dependent inhibitory component by increasing the stimulation frequency from 0.1 to 3 Hz (Fig. 4). To obtain robust Cav3.1 current amplitudes and fast channel recovery from inactivation between pulses, cells were held at −100 mV, and a 3-Hz train of 20-ms test pulses to −20 mV was applied under control conditions and after 2-min incubation in compound. Only a small tonic block component (usually <5%), defined as peak current inhibition during the first pulse after 2-min equilibration in drug, was observed. At 0.1 Hz, negligible current decline due to accumulation of inactivation was observed under control conditions, and 1 μM TTA-A2 had only minor inhibitory effects (3 ± 1 and 14.6 ± 0.6%, respectively). At 3 Hz, Cav3.1 channels accumulate greater levels of inactivation because of the apparent slow rate of recovery from inactivation, leading to an overall current reduction of 32.2 ± 6% in the absence of drug. Addition of 1 μM TTA-A2 significantly enhanced use-dependent inhibition to 69.4 ± 5% at steady state, presumably by stabilizing inactivated channels in between test pulses. Figure 4 summarizes current inhibition under both baseline control conditions and in the presence of TTA-A2 at 0.1 and 3 Hz.

Although low-voltage T-type and high-voltage activated calcium channels share structural similarity, TTA-A2 exhibits remarkable selectivity over Cav1.2 (L-type), Cav2.1 (P/Q-type), Cav2.2 (N-type), and Cav2.3 (R-type) channels in patch-clamp studies. Potential state dependence of Cav1.2 inhibition by 30 μM TTA-A2 was investigated by clamping cells at −90 mV (the majority of channels at rest) and −40 mV (a larger fraction of channels inactivated). Cells were depolarized to +10 mV every 10 s. In contrast to T-type calcium channels, Cav1.2 block by TTA-A2 did not show strong voltage dependence; 40 ± 4.2 and 51.8 ± 7.2% Cav1.2 current inhibition was measured at −40 and −90 mV, respectively (n = 3–5). Hence, recordings from Cav1.2, Cav2.1, and Cav2.3 channels were performed at −90 mV only. At steady state, 30 μM TTA-A2 reduced Cav2.1 whole-cell current amplitude by 35.3 ± 2.8% (n = 3), Cav2.2 amplitude by 56 ± 3.6% (n = 4), and Cav2.3 amplitude by 51.2 ± 4% (n = 4) (Fig. 5). Because of limited solubility of the compound, we were unable to test concentrations above 30 μM. Estimated IC50 values of >30 μM on the tested high-voltage activated calcium channels suggested a ~300-fold selectivity for T-type channels (IC50 of ~0.1 μM) at −80 mV.

Next, we evaluated the effect of TTA-A2 on native T-currents recorded from thalamic relay neurons of the dLGN and STN neurons in rat brain slice preparations (Fig. 6), as these nuclei have been reported to express high levels of Cav3.1 or Cav3.3 channels, respectively (Talley et al., 1999). T-type currents were isolated from high-voltage-activated calcium currents and sodium currents by applying a 400-ms hyperpolarizing prepulse to −100 mV followed by a 200-ms test pulse to −20 mV. IC50 values of >30 μM were determined for Cav3.1 and Cav3.3 channels and were unable to test concentrations above 30 μM. Estimated IC50 values of >30 μM on the tested high-voltage activated calcium channels suggested a ~300-fold selectivity for T-type channels (IC50 of ~0.1 μM) at −80 mV.
pulse from a holding potential of −60 mV in the presence of 250 nM tetrodotoxin. A test potential evoking maximal T-type current amplitudes (typically −35 mV) was determined for each cell using a standard current-voltage relationship protocol. Cells were stimulated every 15 s. Recorded currents were nickel-sensitive and blocked by the nonspecific T-type channel antagonist mibefradil (Kraus et al., 2007). TTA-A2 inhibited T-type currents recorded from both the dLGN (31.3 ± 0.3% at 1 μM, 71 ± 9% at 3 μM; 84 ± 9.1% at 10 μM; n = 3) and the STN (44.5% ± 14% at 1 μM; n = 2). Effects slowly reversed upon washout.

To demonstrate the potential for TTA-A2 to effect neuronal excitability in vivo, the vigilance state of both wild-type mice and those lacking both Ca₃.1 and Ca₃.3 channels were monitored by ECoG/EMG polysomnography during administration of 10 mg/kg TTA-A2. This treatment has been demonstrated to elicit CNS exposures sufficient to affect locomotor activity, feeding, and weight gain in mice with a T_max of 1 h (Uebele et al., 2009a). When administered 1 h before the inactive phase, TTA-A2 acutely suppressed the mean time spent in active wake for up to 2 h following dosing, while promoting delta sleep (Fig. 7). REM also appeared to be reduced, albeit with a delayed time course. As expected, none of these effects was observed in double knockout animals lacking both Ca₃.1 and Ca₃.3 channels.

Discussion

Over the past few years, increasing attention has been focused on the family of low-voltage-activated T-type calcium channels. Functional characterization of T-type channels, including gene knockout and knockdown approaches in rodent models, have furthered our understanding of the physiological role of these channels and have indicated a role for these channels in several neurological disorders. The lack of potent and selective pharmacological tools, however, has so far hampered research efforts to validate T-type channels as targets for drug discovery. Using the recently synthesized antagonist, TTA-A2 (Barrow et al., 2007; Uebele et al., 2009a), we demonstrate the effects of T-type channel inhibition in a cellular context, in vivo on the vigilance state of mice and the therapeutic potential of T-type channel inhibition.

Pharmacological Characterization of TTA-A2. TTA-A2 was found to inhibit all three subtypes of low-voltage-gated T-type channels (Ca₃.1, 3.2, and 3.3) with comparable potencies. Changing membrane resting potentials from −100 to −80 mV enhanced compound potency by ~40-fold, from an IC₅₀ of ~4 μM to 0.1 μM, respectively, indicating state dependence of inhibition. We could further demonstrate a concentration-dependent shift of the Ca₃.1 channel availability curve to hyperpolarized potentials and a slowing of channel recovery from the inactivated state. According to the modulated receptor hypotheses, enhanced T-type channel block by TTA-A2 at depolarized voltages in combination with a shift of the channel availability curve to hyperpolarized potentials can be interpreted as high affinity block of inactivated channels. Furthermore, an increase in channel activation frequency from 0.1 to 3 Hz, to favor the availability of open and/or inactivated channels, revealed an additional use-dependent component of inhibition. Experiments to measure channel recovery from inactivation and use dependence of inhibition had to be performed at −100 mV to obtain robust Ca₃.1 current amplitudes, enable fast channel recovery, and
minimize accumulation of inactivated channels during 3-Hz use-dependence protocols. These experimental conditions provide insight into the mechanism of action of TTA-A2 but may underestimate the compound effects at more depolarized physiological membrane potentials. At such potentials, TTA-A2 most probably causes an even more pronounced delay of channel recovery from inactivation, because of slower dissociation from the receptor, and consequently stronger use-dependent inhibition, consistent with the nanomolar potencies measured at −80 mV and compound-induced effects observed in vivo.

Similar state and frequency dependence has been described for a variety of high-voltage-activated calcium and sodium channel blockers in the past (Hondegem and Katzung, 1984). The exact molecular determinants of TTA-A2 interactions with T-type channels are currently unknown, but previous competition studies in our lab suggested that the binding sites of TTA-A2 and other structurally similar antagonists overlap or interact allosterically with the binding site of mibefradil (Uebele et al., 2009b). Similar to TTA-A2, mibefradil has been reported to preferentially interact with T-type channels in the inactivated state (Martin et al., 2000).

Selectivity of TTA-A2 for T-type calcium channels was previously demonstrated in 170 binding and fluorescent assays, including numerous voltage-activated sodium, calcium, and potassium channels. No activities with IC\textsubscript{50} <10 \textmu M were reported (Uebele et al., 2009a). In the present study we have used electrophysiological patch-clamp experiments at holding potentials similar to neuronal resting membrane potentials (−90 or −80 mV) to demonstrate a ~300-fold selectivity for Ca\textsubscript{3} channels over structurally related high-voltage-activated Ca\textsubscript{1,2}, Ca\textsubscript{2,1}, Ca\textsubscript{2,2}, and Ca\textsubscript{2,3} channels. Frequency dependence of T-channel inhibition by TTA-A2 also suggests potential functional selectivity over other ion channels. This improvement on selectivity clearly distinguishes TTA-A2 from existing blockers such as mibefradil and ethosuximide (Martin et al., 2000; Gomora et al., 2001).

Before testing TTA-A2 in vivo, compound activity was also confirmed on native T-type calcium channel-mediated currents recorded from neurons in the rat subthalamic nucleus and the rat dorsal lateral geniculate nucleus of the thalamus in a brain slice preparation.

**In Vivo Effects of T-Type Calcium Channel Inhibition.** It is well known that T-type calcium channels mediate low-threshold spikes in the thalamus that are essential to sleep-related network oscillations involved in sleep maintenance and sleep/wake transitions (McCormick and Bal, 1997; Cueni et al., 2008). Inhibition of T-currents by depolarization of thalamic neurons or ethosuximide has been shown to affect thalamic oscillations (Huguenard and Prince, 1994; McCormick and Bal, 1997), and mice lacking Ca\textsubscript{3,1} channels display altered sleep/wake patterns because of perturbed thalamocortical activity (Kim et al., 2001; Lee et al., 2004; Anderson et al., 2005). Consequently, it had been proposed that potent and selective T-type channel modulators could serve as pharmacological tools to modulate sleep/wake cycles. To test this hypothesis, the effect of TTA-A2 on the vigilance state of both wild-type mice and Ca\textsubscript{3,1}/Ca\textsubscript{3,3} double knockouts was monitored in ECoG/EMG polysomnographic studies. TTA-A2 acutely suppressed mean time spent in active wake and promoted delta sleep in wild-type mice. Remarkably, none of these effects was observed in the double-knockout animals. In addition to demonstrating the in vivo efficacy of TTA-A2 and its specificity for T-type channels, these results demonstrate that the slow wave sleep-promoting effects of this compound are mediated specifically through Ca\textsubscript{3,1} and/or Ca\textsubscript{3,3} channels. Furthermore, because Ca\textsubscript{3,1} and Ca\textsubscript{3,3} are expressed in cortex, as well as subthalamic and thalamic relay nuclei, whereas Ca\textsubscript{3,2} is present in sensory regions, amygdala and hippocampus (Talley et al., 1999), these findings implicate thalamocortical circuits in the action of TTA-A2.

Interestingly, Lee et al. (2004) demonstrated a reduction of delta (1–4 Hz) and spindle (7–14 Hz) oscillations measured by non-REM electrocorticogram frequency analysis in Ca\textsubscript{3,1} global knockout mice, and Anderson et al. (2005) reported a moderate increase in EEG spectral power within the delta frequency range, when a focal deletion of Ca\textsubscript{3,1} was confined to the rostral midline thalamus. These apparently conflicting findings suggest that the complex neuronal network interactions of multiple nuclei in the thalamocortical system, expressing predominantly Ca\textsubscript{3,1} and Ca\textsubscript{3,3} channels, are not easily understood in genetic models and that the net behavioral effects of an acute pharmacological blockade are not equivalent to constitutive or focal channel ablation. Furthermore, in contrast to an absolute ablation of T-channels in knockout animals, T-channel inhibition by TTA-A2 is dependent on channel state and activation frequency and is therefore modulated by animal state-dependent neuronal network activity in vivo. A potential mechanistic explanation underlying the suppressed active wake and increased delta sleep following TTA-A2 administration may be an increased interburst interval because of slowing of the T-type-mediated initiation of bursting activity of the thalamocortical neuronal network. As previously demonstrated, the inhibition of T-type currents in thalamic reticular nucleus neurons, which establish the pace of recurrent thalamocortical bursts by providing inhibitory input onto the thalamus, can delay the onset of bursting (Huguenard and Prince, 1994) and thus may promote slower oscillatory activity in the network associated with increased non-REM sleep.

T-type channels mediate rebound bursts in cortical and thalamic reticular neurons that are the physiological substrates for spike-wave discharges, the electrographic hallmark of absence seizures. Increased T-type currents have been suggested to elevate the propensity for seizure activity (Khosravani and Zamponi, 2006; Ernst et al., 2009). We have previously shown that selective T-type channel antagonists, including TTA-A2, suppress seizure time in the WAG/Rij rat absence epilepsy model (Shipe et al., 2008; Yang et al., 2008; Uebele et al., 2009b), providing further evidence of the ability of selective T-channel antagonists to modulate thalamocortical network activity.

**Clinical Potential of T-Type Channel Inhibition.** Besides insomnia and epilepsy, pathological changes of membrane potential regulation can lead to thalamocortical dysrhythmia as it was observed in awake patients suffering from neurogenic pain, tinnitus, Parkinson’s disease, schizophrenia, and depression (Llinás et al., 1999). Compared with healthy controls, these patients showed increased thalamocortical oscillations in the \( \theta \)-frequency band. Cortical \( \theta \)-waves (4–8 Hz) usually appear during drowsy or sleeping states. \( \theta \)-Activity is mediated by resonant interaction between the...
thalamus and the cortex, due to the generation of low-threshold old spike bursts by thalamic cells (Crunelli et al., 2006). The abnormal oscillations mediated by T-type calcium channel-triggered low-threshold spikes may be an underlying mechanism for thalamocortical dysrhythmias (Jeanmonod et al., 1996; Llinás et al., 1999). We have shown that thalamocortical network activity can be modulated by TTA-A2, and that these effects depend on the presence of T-type channels. Hence, the termination of abnormal thalamocortical oscillations with selective T-type calcium channel antagonists could offer an interesting novel therapeutic approach to treat a diversity of diseases associated with thalamocortical dysrhythmias.

Conclusions

TTA-A2 is a potent, state-dependent, and highly selective T-type calcium channel antagonist. Using TTA-A2 we were able to demonstrate the effects of pharmacological T-type calcium channel inhibition on thalamocortical activity in vivo, as assessed in mouse polysomnographic experiments and previously reported rat absence epilepsy studies. TTA-A2 represents an invaluable tool for elucidating the true physiological roles of T-type calcium channels and for exploring these channels as drug targets for a variety of human neurological diseases, including sleep disorders and epilepsy.

References


Address correspondence to: Richard L. Kraus, Department of Neuroscience, Merck Research Laboratories, 770 Sumneytown Pike, West Point, PA 19486. E-mail: richard_kraus@merck.com